ASPECTS OF DIETARY MANAGEMENT AND DYNAMICS OF THE FAECAL MICROBIOTA OF HORSES AND PONIES (*Equus caballus*) IN NEW ZEALAND

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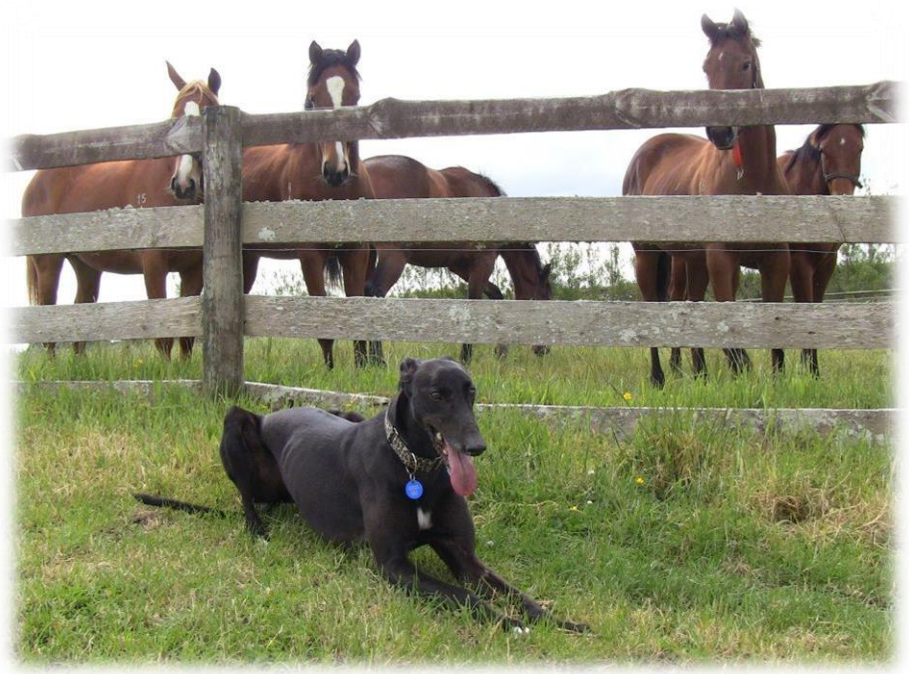
This thesis is dedicated to the wonderful people and animals who have taught me so much about life…

especially to beloved Ricki



ABSTRACT

The aim of this thesis was to explore aspects of the dietary management of horses and ponies in New Zealand, and to investigate the association between dietary management and faecal microbiota. To achieve this aim, a series of observational and intervention studies were conducted.

The first two studies were cross-sectional surveys of feeding, health and management practices, which showed that most horses and ponies in New Zealand were managed continuously on pasture all year round, with no seasonal differences in the hours allowed for grazing. In addition to pasture, many owners fed their animals a combination of premixed feeds, cereals (oats) and conserved forages. Most horses and ponies kept on pasture were reported to be healthy. Among nutrition-related health issues reported by the owner, obesity, colic, laminitis and grass staggers were most commonly reported (12-14%). Using a standard body condition scoring chart, 22% of owners indicated that their horse or pony was overweight (scored ≥ 7 on a 1-9 scale). Horses and ponies kept on pasture maintained body weight and a higher body condition (median score of 6 on a 1-9 scale) through spring and autumn. A high proportion of pony breeds were observed in this population, and these ponies remained 'fat' despite the seasonal fluctuation in the quantity and quality of pasture. Owners tended to underestimate the body condition of their horses, especially ponies, and this finding indicated why a higher percentage of overweight animals may be present in the Pony Club population.

The next two studies were observational investigations that characterised the faecal microbiota of forage-fed horses. The faecal microbiota in a cohort of yearling Thoroughbred horses that were abruptly transitioned from an ensiled chopped forage-based diet to pasture was diet-specific and responded rapidly to dietary change within four days. The faecal microbiota profile was dominated by two phyla, Firmicutes and Bacteroidetes, which comprised of several bacterial genera. The abundance of bacterial genera fluctuated over the three-week observation period, when kept at pasture. Similarly, the faecal microbiota of a cohort of mature adult Thoroughbred and Standardbred horses kept on pasture was diet-specific. The abundances of the bacterial genera were influenced by the nutrient composition of the pasture, which was also correlated with seasonal changes in climate (rainfall and temperature) over the one year observation period. This latter finding indicated that the fluctuations observed in the previous study may also be due to changes in pasture composition.

The inclusion of hay in the diet appeared to buffer the changes occurring in the faecal microbiota as a result of the seasonal fluctuations in pasture composition, but there was also a large degree of variation between individual horses.

The final study was a randomised controlled trial using adult Thoroughbred horses that were kept in loose boxes and fed four forage-based diets. The first phase of the trial identified that the mean retention time of digesta was associated with the dry matter intake of the feed consumed. There was a significant difference in the quantity of feed consumed by individual horses, which appeared to be driven by the moisture content in the forage diets. The second phase of the trial showed that the population of the faecal microbiota was resilient following abrupt dietary transition between four forage-based diets. These findings indicated why the horses in the previous study may have maintained body weight and condition, despite the seasonal fluctuation in the quantity and quality of pasture.

This thesis highlights the complexity of the equine faecal microbiota, and demonstrates that the relationship of dietary dry matter intake and mean retention time of digesta in the gastrointestinal tract influences the population dynamics of the faecal bacterial community.

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Karlette Anne Fernandes

Palmerston North, New Zealand

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LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
BCS	Body condition score
BW	Body weight
BWA	Burrows Wheeler aligner
CFU	Colony forming units
CH4	Methane
CHO	Carbohydrates
CNS	Cresty neck score
DE	Digestible energy
DM	Dry matter
DMI	Dry matter intake
ECS	Equine Cushing's disease
eHGM	Equine hindgut microbiome
EMS	Equine metabolic syndrome
ESNZ	Equestrian Sports New Zeaalnd
F:B	Firmicutes: Bacteroidetes
FE	FiberEzy®
FW	Faecal weight
GE	Gross energy
GI	Gastrointestinal
HNF	High Nutritional Fiber
IQR	Interquartile range
IR	Insulin resistance
MEGA	Molecular Evolutionary Genetics Analysis
MJ	Megajoules
MRT	Mean retention time
MUAEC	Massey University Animal Ethics Committee
NC	Neck circumference
NCBI	National Centre for Biotechnology Information

NDF	Neutral detergent fibre
NFC	Non-fibre carbohydrates
NSC	Non-structural carbohydrates
NSC	Non-structural carbohydrates
NZCAC	New Zealand Companion Animal Council
NZPCA	New Zealand Pony Clubs Association
OR	Odds ratio
OTU	Operational taxonomic units
PAM	Post-administration of markers
PAST	Paleontological Statistics
PC	Pony club
PCoA	Principal coordinates analysis
PCR	Polymerace chain reaction
QIIME	Quantitative Insights into Microbial Ecology
SD	Standard deviation
SRA	Sequence read archives
TDN	Total digestible nutrients
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USDA	United States Department of Agriculture
VFA	Volatile fatty acids
WSC	Water soluble carbohydrates

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Background

The horse is an herbivorous animal with a gastrointestinal tract that has evolved to digest small quantities of starch and sugars via enzymatic digestion in the foregut, and large quantities of fibre-rich plant material via microbial fermentation in the hindgut (Frape, 2010). Therefore, the horse is physiologically dependent on the fermentation of structural carbohydrates, such as cellulose and hemicellulose, as the primary source of energy. Modern horse management typically involves confinement, restricted amounts of free-exercise, feeding regimens that incorporate energy-dense diets for athletic performance, reduced forage intake, abrupt dietary changes, or access to improved pastures. These management practices are in stark contrast to the natural ecology of feral horses (free-grazing on poor quality pasture), and hence, disruptions in the physiological capability of digestion and fermentation in the gastrointestinal tract are commonly reported (Goodwin *et al.*, 2002, Richards *et al.*, 2006). A number of disorders appear to be associated with modern dietary management, mainly obesity, laminitis and colic (Anonymous, 2000 and 2005).

In New Zealand, the year-round availability of pasture makes grazing a common feeding practice to provide all or the majority of a horse's diet (Williamson *et al.*, 2007, Hirst, 2011). The pastoral system in New Zealand provides low-cost feeding to support moderate levels of performance, and typically comprises perennial ryegrass (*Lolium perenne*) as the dominant grass species with a lesser contribution from legumes (clover) and weeds (Holmes *et al.*, 1987, Hoskin and Gee, 2004, Waghorn and Clark, 2004). However, the energy content of pasture varies seasonally and has been suggested to be associated with obesity and laminitis in some horses, and especially ponies (Longland *et al.*, 1999, Litherland *et al.*, 2002).

The management of horses in the New Zealand racing industry is well documented (Rogers *et al.*, 2007, Williamson *et al.*, 2007), but information on the feeding and management of leisure horses and ponies is lacking. Information on the incidence of obesity and other nutrition-related diseases in leisure horses and ponies in New Zealand is largely anecdotal. Hence, studies are required to explore the demographics, feeding, health and management of leisure horses and ponies in New Zealand.

Alterations in feed intake either as a result of seasonal pasture availability or other changes to feeding regimes may cause changes in the gastrointestinal ecosystem, and these changes are believed to have consequences on the health of the animal (Julliand *et al.*, 2001). The incorporation of large quantities of cereal grains with reduced proportions of forage in the diet, or the accelerated pasture intake associated with restricted pasture access, could lead to an increased amount of fermentable substrate reaching the hindgut. Rapid fermentation of carbohydrates results in a reduction in hindgut pH and disturbances in the microbial populations, which place the horse at a greater risk of developing intestinal dysfunction (Julliand *et al.*, 2001, Medina *et al.*, 2002, Milinovich *et al.*, 2008). Evidence suggests that the population of hindgut microbiota are highly sensitive to dietary changes, and maintaining a balance in the hindgut microbial populations has been suggested for sustaining optimal gastrointestinal health (Sadet-Bourgeteau and Julliand, 2010).

Faecal pH and microbial culture have been previously used to monitor changes in the equine hindgut and faeces in response to dietary changes (Berg *et al.*, 2005, Hastie *et al.*, 2008, Yamano *et al.*, 2008, Willing *et al.*, 2009, van den Berg *et al.*, 2013). More recently, faecal samples have been used to profile the microbial diversity in the hindgut by molecular analysis of PCR-amplified 16S ribosomal RNA gene sequences using next generation sequencing technology (Schuster, 2007, Costa and Weese, 2012).

It is essential to understand the dynamics of the hindgut microbiota when developing feeding strategies for horses and ponies that may be at risk of developing gastrointestinal disturbances (Vörös, 2008, Willing *et al.*, 2009). However, there is currently little information on the complex relationship of dietary management and the microbial populations in the hindgut or faeces, and the variation in the microbiota between individual horses (de Fombelle *et al.*, 2001, Milinovich *et al.*, 2006, Steelman *et al.*, 2012). Hence, studies are required to quantify the faecal microbiota in different groups of horses managed under different dietary conditions.

1.2 Project design and objectives of the thesis

This thesis investigates different aspects of dietary management and the effects this has on the faecal microbiota of horses. A series of studies describe the dietary management of horses and ponies in New Zealand, quantify the diversity of the faecal microbiota of horses fed different forage diets, and establish if there are significant associations between the dietary management and populations of faecal microbiota.

An in depth literature review is presented in Chapter 2 and covers three major areas: 1) modern horses and their management, 2) the equine digestive system, and 3) factors that affect the hindgut microbiome. Gaps in our current knowledge of these areas are identified, followed by a statement of the research questions and the specific aims and hypotheses of each experimental chapter of this thesis.

The Chapters 3-8 are a series of studies, presented in the form of original research articles published in (or submitted to) peer-reviewed academic journals. The first two studies (Chapters 3 and 4) use online and face-to-face survey tools to describe the feeding, management, and health of leisure horses and ponies in New Zealand, and investigate the changes in dietary management and body condition of horses and ponies kept on pasture over two seasons. These studies quantify baseline information within the Pony Club horse population. The common feeding and management practices identified in Chapters 3 and 4 are incorporated in the experimental design of the four subsequent studies reported in this thesis; two observational studies (Chapter 5 and 6) and two intervention studies (Chapters 7 and 8), which investigated the relationship between dietary management and the faecal microbiota of horses. The experimental studies utilise non-invasive next generation sequencing techniques to characterise the faecal microbiota of normal horses kept on typical New Zealand pasture, examine the variability in the faecal microbiota between horses and diets, and investigate changes that occur in the faecal microbiota population following changes in dietary management and the dynamics of the process.

Lastly, in Chapter 9 the outcomes of this research are summarised, implications for the equine industry are discussed, and future directions for research are indicated. A list of the publications produced from this doctoral thesis are provided at the end of the thesis.

The overall objectives of the thesis are as follows:

1) To examine aspects of dietary management of horse and ponies in New Zealand.
    a. To describe the demographics, feeding, health and management of Pony Club horses in New Zealand.
    b. To evaluate and compare the body condition and measures of adiposity in Pony Club horses at two time points (spring and autumn).
    c. To investigate if owners accurately estimate the body condition of their Pony Club horses using a standard body condition scoring chart.

2) To examine population dynamics of the faecal microbiota of forage-fed horses in New Zealand, using next generation sequencing techniques.
    a. To characterise the normal profile of the faecal microbiota of horses kept on pasture in New Zealand.
    b. To investigate whether the faecal microbiota of horses are diet-specific.
    c. To investigate the faecal microbiota profiles of horses fed the same diet and assess the degree of inter-horse variation.
    d. To investigate the variation in faecal microbiota population in normal horses kept on pasture and correlate these to seasonal differences in pasture composition.
    e. To investigate the relationship between feed intake and digesta transit time in horses fed different forage-based diets.
    f. To investigate the effects of abrupt dietary transition on the population dynamics of the faecal microbiota of horses fed different forage-based diets.

CHAPTER 2

A REVIEW OF LITERATURE

(Dietary Management and the Equine Hindgut Microbiome)

CHAPTER 2: A REVIEW OF LITERATURE

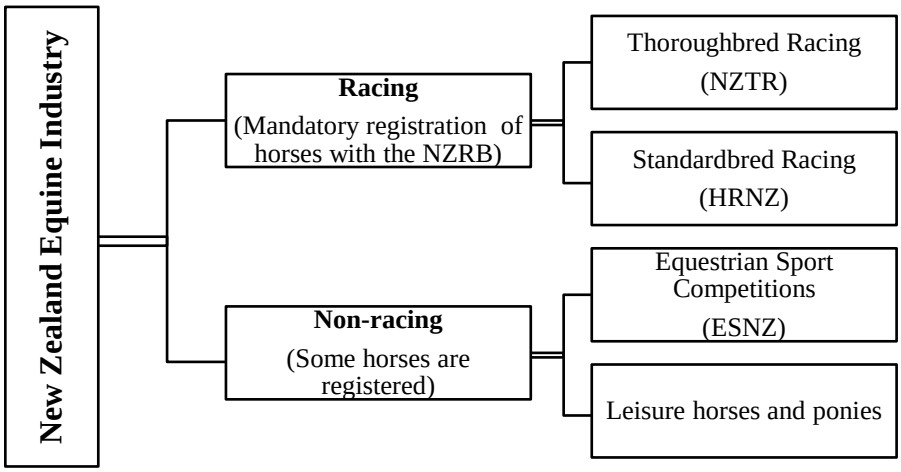
This chapter provides an overview of the New Zealand equine industry, general feeding and management practices, and the health problems associated with diet and management of horses and ponies. Further, it highlights the important features of the equine gastrointestinal anatomy and digestive physiology, and the significance of the hindgut microbiota. Lastly, factors that affect the hindgut microbiome, and the alterations that occur in the microbiota population due to dietary interventions or disease are reviewed.

2.1 Modern horses and their management

2.1.1 The equine industry

In New Zealand, the equine industry can be divided into two sectors, racing and non-racing (Figure 2.1). The racing industry includes Thoroughbred racing (NZTR - New Zealand Thoroughbred Racing) and Standardbred racing (HRNZ - Harness Racing New Zealand), both of which focus on the production (breeding) and the training of horses for racing performance (racing). Both these sectors are governed by the New Zealand Racing Board (NZRB), which mandates registration of all racehorses with the racing board (Anonymous, 2013). There are ~33,000 horses registered with the racing industry in New Zealand, which includes horses kept for racing and breeding (Rogers and Firth, 2005, Rogers and Vallance, 2009).

Figure 2.1. Structure of the New Zealand Equine Industry.



Adapted from Rosanowski (2012)

The non-racing sector of the New Zealand equine industry is relatively less organised than the racing sector. It comprises of horses competing in International Equestrian Federation (FEI) sports such as dressage, show jumping, eventing, endurance, and para-equestrian activities, and horses and ponies that are kept for leisure purposes (pleasure riding, trekking, hacking, Pony Club, trail riding, hunting, showing, field games, etc.), and companionship (pets or therapy animals). While the sporting sector is fairly organised and governed by Equestrian Sports New Zealand (ESNZ), most leisure horses and ponies remain unregistered and maybe under-represented in the census of the national horse population (Rosanowski, 2012).

Due to the large numbers of unregistered horses, the size of the New Zealand horse industry is currently unknown, but is estimated to be between 100,000 to 120,000 horses (Rogers and Vallance, 2009). Given that ~33,000 horses are registered in the racing and breeding industry, this leaves ~87,000 horses classified as sport and leisure horses. Rosanowski *et al.*, (2012b) examined the demographics of horses kept on non-commercial horse properties in New Zealand using the AgriBase™ database - a farm search online system (www.searchagribase.asurequality.com), and found that the majority of respondents kept their horses for recreation activities (51%), equestrian sport competitions (40%), and companionship (pets) (15%). These findings are in agreement with other studies in Europe and the United States of America (Anonymous, 2006, Hotchkiss *et al.*, 2007, Liljenstople, 2009), and indicate that currently, most horses are kept for non-racing purposes.

In New Zealand, there is a high level of domestic equestrian sport participation, with nearly 5% of New Zealanders reported to have participated in equestrian sport (Anonymous, 2011). The number of horses participating in competitions is estimated to be ~5,000 (<7% of the total sport and leisure horse population) (Rogers and Firth, 2005), leaving ~82,000 (>90%) horses that could potentially be classified as leisure horses (including ponies). In 2016, a general survey by the New Zealand Companion Animal Council Inc. (NZCAC) estimated that ~2% New Zealand households keep an average of three horses or ponies as companion animals, and the equine population was estimated to comprise of ~116,000 animals (Anonymous, 2016). These numbers indicate that leisure horse and ponies constitute the largest proportion of the New Zealand equine industry.

2.1.2 Feeding and management

Horses are typically managed in stables and fed forage-based compound diets (intensive management), allowed to graze on pasture (pasture-based management), or

managed using a combination of both the management systems (semi-intensive management). The choice of management depends on the availability of land and feed resources, the climate of the geographical region, and the utility purpose of the animal (performance versus leisure). In general, racehorses in New Zealand are managed intensively in stables, competition horses are managed on a semi-intensive management system, and leisure horses and ponies may be managed using a variety of management practices (Stowers *et al.*, 2009, Rogers and Firth, 2005, Rogers *et al.*, 2007, Rosanowski *et al.*, 2012b, Verhaar *et al.*, 2014).

The feeding and management of racehorses has received significant research attention, but there has been limited research involving the non-racing horse population (Huntington and Jenkinson, 1998, Bryden *et al.*, 2001, Pagan, 2004, Hotchkiss *et al.*, 2007). Horses under intensive management generally have low levels of activity due to confinement, and are provided a restricted diet, which is fed as concentrated meals containing high-starch cereals and high-fibre conserved forages. High-fibre forage diets can fail to meet the energy requirements during intense work, thus horses are often fed variable amounts of concentrates to meet performance needs (NRC, 2007).

In New Zealand, intensive management of racehorses during training is similar to practices in other major racing countries (United States of America and Australia), and involves long periods of confinement with limited or no access to pasture. Most horses are confined in an area $\leq 5 \times 5$ m for ≥ 12 hours/day, and are only turned out on pasture for short grazing periods, or fed cut pasture in stables. A typical racehorse diet comprises concentrate meals available at specific feeding times, and conserved forage available *ad libitum* (Rogers *et al.*, 2007). Oats are the most popular cereals fed to horses in New Zealand and Australia (Huntington and Jenkinson, 1998). However, the composition of the diet may vary depending on individual horse requirements or due to trainer choice (Huntington and Jenkinson, 1998, Williamson *et al.*, 2007). When out of training, racehorses are generally spelled on pasture (Huntington and Jenkinson, 1998, Williamson *et al.*, 2007). In comparison to the well-documented feeding and management of New Zealand racehorses, scientific literature on the management of competition and leisure horses (and ponies) is limited, with reports that horses are mostly kept on pasture and managed in a variety of ways (Rosanowski *et al.*, 2012b, Verhaar *et al.*, 2014).

The New Zealand leisure and competition horse industry relies on the year-round availability of pasture as the primary basis for feeding horses (Hoskin and Gee, 2004, Rogers

et al., 2007, Verhaar *et al.*, 2014). Good quality pasture can provide most of the nutritional needs of a horse, and may also be beneficial by providing additional protein, vitamins, and non-structural carbohydrates for healthy digestive function (Hoskin and Gee, 2004). Advantages of grazing horses on good quality pasture are low cost feeding, year round availability and the low risk for digestive and behavioural problems when compared to stabled horses (McGreevy *et al.*, 1995, Huntington and Jenkinson, 1998). However, the composition of pasture may vary regionally, and across seasons, because it is influenced by changes in soil and climatic conditions (Grace *et al.*, 2002). Furthermore, the high moisture content in pasture (~80% water) results in significant gut fill, and may limit the horses' ability to consume sufficient quantities to meet their energy requirements when under intense work (such as racing and competition).

The National Research Council (2007) suggests that a horse in light work should consume a diet containing 80% roughage and 20% concentrate. Roughage comprises forage either in the fresh or conserved form. Fresh forage may be pasture grass grazed in paddocks, or cut and fed to horses in stables. Lucerne is a common legume forage fed in stables in fresh or conserved form. Conserved forage may be dried and offered as hay or chaff, or fermented. Concentrates are feeds that are high in energy and are highly digestible in the small intestine of the horse (pre-caecal). These may include whole grains or processed cereals, grain-by-products, and commercially prepared feeds (including a combination of processed grains that are prepared into a textured, extruded, or pelleted form). Supplementary feeds may be fed to provide additional protein, fats, minerals and vitamins. To provide additional energy for a horse maintained on pasture or in heavy or intense work, the proportion of concentrates is often increased with a compensatory decrease in the roughage component.

2.1.3 Health problems associated with dietary management

Modern practices of feeding and management have subjected the domestic horse to an array of health problems that are not typically experienced by feral horses. The intensive or restrictive management of horses and the feeding of mixed roughage and concentrate diets, with characteristically reduced fibre consumption, can affect the digestive function, and impair the health and performance of horses (Frape, 2010).

In the racing industry, there is a high-energy requirement to support optimum athletic performance, which is achieved by feeding a high-grain low-forage diet. Typically, this can result in the increase of high-energy grain rations and decrease of forage incorporated in the

diet (Frape, 1994). Grains are high in starch and soluble carbohydrates and are enzymatically digested in the small intestine, whereas forages are high in fermentable carbohydrates and are digested via microbial fermentation in the hindgut. There is strong epidemiological evidence that confinement, restricted feeding times, and diets low in roughage and high in hydrolysable carbohydrates, significantly increase the risk for intestinal dysfunction in horses (Archer and Proudman, 2006, Milinovich *et al.*, 2006, Willing *et al.*, 2009).

Dietary fluctuations, especially the incorporation of high-starch ingredients into feed rations and thus increase in non-structural carbohydrates, have been associated with alterations in faecal pH and the population of hindgut microbiota in horses (de Fombelle *et al.*, 2001, Drogoul *et al.*, 2001, Julliand *et al.*, 2001, Willing *et al.*, 2009, Muhonen *et al.*, 2010, van den Berg *et al.*, 2013). Additionally, there appears to be significant individual variation in hindgut pH and microbial populations between horses, and some authors have suggested that because of this individual variation, certain horses have a microbial population predisposes them to a number of nutrition-related health complications (Milinovich *et al.*, 2006, Al Jassim and Andrews, 2009, Willing *et al.*, 2009, Steelman *et al.*, 2012, van den Berg *et al.*, 2013). Changes in dietary management and the population of hindgut microbiota may trigger the development of conditions such as laminitis, colic, gastric ulcers, diarrhoea due to acidosis and stereotypic behaviours in horses, which may negatively affect horse performance (White, Cohen *et al.*, 1999, Hudson *et al.*, 2001, Milinovich *et al.*, 2006, Steelman *et al.*, 2012, Venable *et al.*, 2013, Weese *et al.*, 2015).

Modern 'lush' pastures that are increasingly available to domestic horses and ponies are comprised of improved grasses and legumes that have been developed for production animals such as ruminants, and therefore have a much higher energy content (in particular, the soluble sugar content that increases the energy content) than the fibrous type of grasses that horses evolved on. Modern management practices place horses and ponies in unnatural confined areas that restrict the area of grazing activity with paddock fences while providing easy to find, high quality forages. Native breeds of ponies are popular animals for leisure riding (Garber, 1998, Rosanowski *et al.*, 2012b). These ponies generally have a smaller body size and appear to be adapted to harsh environments and poor dietary conditions (Yamano *et al.*, 2008). Their strong evolutionary selection for efficient feed conversion enables them to maintain body condition even with low caloric intake (Kawai *et al.*, 2004). For these animals, the combination of confinement, decreased work expectations, improved forage qualities, increased provision of feed and modified husbandry practices to limit energy losses through

thermoregulatory demands, have served to promote the year-round maintenance of a positive energy balance leading to excessive body weight (Argo, 2009).

Another aspect to the weight management issue, is that many horse and pony owners fail to recognise changes in body weight and condition, and tend to provide calories in excess of their horse's energy requirements (Robin *et al.*, 2013). A standardised body condition scoring (BCS) system has been used to assess body condition in horses and ponies (Henneke *et al.*, 1983). However, the capability of this scoring system to accurately reflect the regional distribution of adiposity observed in ponies, and the ability of untrained owners to accurately assess BCS has been called into question (Martinson *et al.*, 2014).

Excessive body weight (obesity) has become a major health and welfare issue in the equine industry (Anonymous, 1997, Geor *et al.*, 2007, Thatcher *et al.*, 2008, Dugdale *et al.*, 2010, Menzies-Gow *et al.*, 2010, Argo *et al.*, 2012), and is similar to the global epidemic of human and companion animal obesity (German, 2006, Johnson *et al.*, 2009). A recent study showed that horses used for recreation were more likely to have a higher BCS compared to horses used for breeding (OR = 3.07) and instruction (OR = 2.06) (Visser *et al.*, 2014). A combination of genetic susceptibility, increased availability and feeding of high-energy feeds and a decreased requirement for physical activity resulting in an imbalance between energy intake and expenditure, have been identified as underlying reasons for the problem (Kopelman, 2000).

Management strategies to combat this problem include provision of smaller paddocks to restrict the pasture access and energy intake of obese ponies (Harris, 2012). However, this also results in decreased voluntary exercise, and therefore, the imbalance in energy intake and expenditure persists. Additionally, observational studies have revealed that ponies maintained on restricted pasture tend to increase their voluntary feed intake and the rate of feed intake, and therefore are able to maintain body condition (Dugdale *et al.*, 2010, Glunk *et al.*, 2013a). Furthermore, severe dietary restriction is not recommended for horses and ponies, as starvation can trigger the development of hyperlipaemia, a potentially fatal sequel to rapid lipolysis, to which obese ponies are particularly susceptible (Hughes *et al.*, 2004).

The major health problems associated with dietary management in horses are laminitis, colic, gastric ulcers, acidotic diarrhoea, and stereotypic behaviours. A short description of each condition is given below.

Laminitis is a metabolic disorder caused by inflammation of the sensitive laminae of the horses' foot (Pollitt, 2004). The aetiology is multifactorial, but the most common causes are due to abrupt changes in dietary management, a high level of soluble carbohydrate in the diet (carbohydrate-associated laminitis), and underlying metabolic disorders such as obesity, equine metabolic syndrome and insulin resistance (Geor, 2008). A significant shift in microbial populations in the hindgut has been observed preceding an episode of carbohydrate-induced laminitis (Milinovich *et al.*, 2010).

Colic is characterised by clinical signs of abdominal pain due to inflammation, distension or obstruction in the gastrointestinal tract (Durham, 2009). The aetiology of colic is also multifactorial, but the most common risk factors are internal parasites, a change of diet, dietary management, housing conditions, a lack of access to pasture and water, increasing exercise, and transport (Archer and Proudman, 2006). Factors that cause rapid fermentation and excessive build-up of gasses in the hindgut and could lead to distension of the digestive tract and the release of endotoxins, along with changes in the gut microbial populations immediately preceding an episode of colic have been described (Venable *et al.*, 2013).

Gastric ulcers (lesions in the stomach) are a result of gastric mucosal damage from excessive gastric acid and pepsin (Bell *et al.*, 2007). The incidence of ulcers is more prevalent in horses that are confined during training and competition with an interrupted availability of feed, and those regularly administered with anti-inflammatory drugs. Feeding grain or concentrates rich in non-structural carbohydrates, bolus/meal feeding, and feed deprivation for prolonged periods all increase the duration of exposure of the non-glandular stratified squamous epithelium of the stomach to acid, potentially causing gastric ulcers (Andrews *et al.*, 2005). Gastric secretion is continuous in horses and the secretion rate is greater when they are fed grain compared to hay (Nadeau *et al.*, 2003). Therefore, a high grain diet may increase the incidence of ulceration due to reduced chewing required for grain consumption compared to hay and therefore limited salivary secretion and thus the buffering effect of bicarbonate. There is a significant link in the interval between feeding forage and the risk of gastric ulceration, wherein horses with forage feeding intervals of more than 6 hours have 3.9 times greater likelihood of developing gastric ulcers (Luthersson *et al.*, 2009). Horses maintained solely on pasture are less likely to develop gastric ulcers (Al Jassim and Andrews, 2009).

Diarrhoea due to acidosis is associated with an accumulation of lactic acid and a decrease in hindgut pH. Ingested starch that exceeds the capacity of enzymatic digestion in

the small intestine enters the large intestine, where it is rapidly fermented, thereby increasing volatile fatty acids (VFAs) and lactate production. An acidic environment favours the rapid proliferation of lactic acid producing bacteria, resulting in increased lactic acid production and a further decline in the pH. Changes in microbial populations and increased permeability of the intestinal mucosa and endotoxin levels are also observed in the horse. Thus, lactic acidosis is often accompanied by laminitis (Al Jassim and Andrews, 2009).

Stereotypic behaviours in horses (such as crib biting, pacing, wind sucking, etc.) are commonly associated with intensive management, due to confinement and the reduced roughage intake (McGreevy *et al.*, 1995). These behaviour problems may also be associated with disturbances in the microbial population in the hindgut (Destrez *et al.*, 2015).

2.2 The equine digestive system

The diet and living conditions of domestic horses have changed to the extent that some of these conditions may no longer be a good fit with the evolutionary physiology of digestion in the species. The digestion of feed and absorption of nutrients are important processes required for optimal health and performance of the animal. Optimum health is dependent on healthy digestive function maintained by microbial populations in the gastrointestinal (GI) tract. Therefore, before exploring the intestinal microbiota and factors that influence microbial populations, it is important to understand the normal digestive function of the horse. This section provides an overview of the anatomy and physiology of the equine digestive tract, with a focus on hindgut fermentation.

2.2.1 Differences in digestion between horses and ruminants

The horse has evolved as a grazing herbivore with a specialised GI tract capable of utilising plant material. Under feral conditions, horses graze for 12-18 hours per day, on a variety of grasses (65%), shrubs (25%) and minimal amounts of plant stems (10%) (Salter and Hudson, 1979, 1982), with few options to consume feeds containing high starch or sugar content (non-structural, hydrolysable carbohydrates). By continuously grazing for long periods, horses trickle feed on forages that are high in fibre content (structural carbohydrates such as cellulose and hemicellulose) and consume sufficient quantities to meet daily energy requirements for maintenance of body weight (Franzen, 2006, Ringler *et al.*, 2009). However, these plant structural carbohydrates cannot be digested by enzymes produced in the

mammalian GI tract (Hume and Warner, 1980). Therefore, in common with other herbivore animal species, the GI tract of the horse has evolved anatomically and is colonised by microbial species to allow digestion of structural carbohydrates to meet their digestive needs.

Although equids and bovids are similar in many aspects of their biology (both are herbivores and depend on microbial fermentation for digestion of plant fibre) (Fritz *et al.*, 2009), the anatomy of the GI tract and digestive physiology of horses differs significantly from ruminants (Janis, 1976, Cymbaluk, 1990). Ruminants have multiple pre-gastric fermentation chambers (rumen, reticulum, omasum) located proximal to the abomasum (true stomach) and the small intestine (major site of nutrient absorption). In contrast, horses are monogastric animals (similar to pigs), with an expanded hindgut that is comprised of two post-gastric fermentation chambers (caecum and colon) located distal to the small intestine (Frandson *et al.*, 2009). Hence, horses are referred to as hindgut fermenters.

This unique digestive system provides horses with the ability to digest and absorb small quantities of hydrolysable carbohydrates and amino acids in the small intestine (chemical and enzymatic digestion), followed by digestion of the remaining plant material (cellulose and hemicellulose) via microbial fermentation in the hindgut (Demment and Van Soest, 1985, Al Jassim and Andrews, 2009). The caecum and large colon are described as a fermentation vat, analogous to the rumen and reticulum in cattle. However, the relative size of these fermentation chambers and their contents comprise ~40% of the body weight in ruminant cattle compared with only 15% in horses (Janis, 1976). The feeding behaviour and location of the fermentation vat is also different in ruminants and horses (i.e. intermittent grazing and rumination with pre-gastric fermentation versus continuous grazing and post-gastric fermentation, respectively). Accordingly, ruminants can digest cellulose more efficiently and need to eat less than horses on a per weight basis.

The digestive efficiency of caecal fermentation in horses is only 70% as efficient as ruminant fermentation (Franzen, 2006), and this difference is mainly because many products of digestion that occur beyond the absorptive phase of the small intestine in horses, cannot be assimilated (apart from volatile fatty acids, VFAs) (Hintz *et al.*, 1978, Hintz and Cymbaluk, 1994). Ruminants are able to utilise the bacterial protein created during fermentation by enzymatic digestion in the small intestine (located distal to the rumen). Thus, ruminants can use a variety of nitrogen sources from the feed consumed, whereas horses are unable to digest

most of the bacterial protein produced during fermentation, which is excreted in the faeces (Cymbaluk, 1990).

To compensate for the lowered efficiency in digestion, horses have adopted a strategy of high food intake (by grazing for the major part of their day) with fast digesta passage rate to meet their nutritional requirements (Frape, 2010, Janis *et al.*, 2010). For example, a free ranging horse will graze 10-16 hours per day with a digesta transit time of 21-48 hours. A similar sized ruminant will graze 7-8 hours per day with a 90-hour digesta transit time, when feeding on the same high fibre pasture (Pearson *et al.*, 2006, Frape, 2010). This feeding strategy, allows horses to thrive on dry, poor quality, cellulose-rich plant matter, unlike ruminants (Cuddeford *et al.*, 1995, Pearson *et al.*, 2001). Moreover, the lesser weight of the digestive tract and its contents in horses and the weight positioned further back in the animal (i.e. better centre of gravity), are evolutionary adaptations of the GI tract beneficial for the survivability of a cursorial type of animal with a well-developed flight instinct to evade prey by running (i.e. the weight of the GI tract being inversely proportional to speed) (Sjaastad *et al.*, 2010). Therefore, horses are not 'inferior' to ruminants, their GI tract merely represents an adaptation to a different ecology than that of ruminants (Janis, 1976).

2.2.2 Anatomy and physiology of the equine digestive system

The primary functions of the digestive system are prehension, mastication, digestion and absorption of food, and finally elimination of solid wastes (faeces). The GI tract of a horse is divided into the foregut (small intestine) and hindgut (large intestine), along with associated organs such as the liver and pancreas (Figure 2.2). Each compartment of the GI tract has a unique and significant role in converting complex feeds into substrates (nutrients) that can be absorbed and metabolised by the body tissues (Stevens and Hume, 1998). The hindgut comprises the major proportion of the equine GI tract where majority of digestion occurs via microbial fermentation (Table 2.1).

Figure 2.2. Detailed schematic diagram of the equine gastrointestinal tract.

Al Jassim and Andrews (2009)

Table 2.1. Comparison of the length, volume, and proportion of various compartments of the equine gastrointestinal (GI) tract.

Type of digestion	GI compartment (length in meters)	Capacity (volume in litres)	Proportion of total GI tract (%)
	Foregut		
Chemical	Stomach (<0.5 m)	8-15 L	8%
Enzymatic	Duodenum, jejunum, ileum (21 m)	68 L	30%
Microbial	***Hindgut***		
	Caecum (12 m)	28-36 L	15%
	Large colon (ventral and dorsal) (3-3.6 m)	86 L	38%
	Small colon (3-3.6 m)	16 L	9%

Adapted from Popesko (1978)

2.2.2.1 Foregut anatomy and pre-caecal digestion

The mouth and mechanical digestion

The mouth and oesophagus are responsible for the initial breakdown and swallowing of ingested food. The lips, tongue and teeth of the horse are ideally suited for prehension, ingestion and alteration of the physical form of feed by mastication. Since fibre-rich forages constitute a major part of the horses' diet; horses have developed an effective masticatory (chewing) apparatus to efficiently utilise the feed consumed (Frape, 2010, Janis *et al.*, 2010). Horses have forward positioned incisors to graze close to the ground and through a complex chewing mechanism (vertical, horizontal and rolling movements of the jaws), the occlusal surfaces of the cheek teeth effectively grind the ingested feed to smaller particle sizes and break down the less digestible outer coverings of grains and forages, for the effective utilisation of fibrous feeds (Janis, 1988, Jams *et al.*, 1994).

Moistening of the ingested feed with saliva marks the beginning of the digestive process. Equine saliva contains >99% water along with small amounts of other components including sodium, potassium, chloride, calcium, magnesium, bicarbonate, phosphate, urea and protein (Alexander, 1966, Eckersall *et al.*, 1985). Equine saliva contains limited amounts of digestive enzymes. The mean concentration of α-amylase (0.44 U/ml) in equine saliva is

considered insufficient to digest starch, resulting in negligible oral starch disappearance (Julliand *et al.*, 2006, Varloud *et al.*, 2007b). Furthermore, equine saliva contains less than 10 CFU (colony forming units) of total bacteria/ml and there are no specific data regarding the activity levels and properties of salivary lysozymes in horses (Varloud *et al.*, 2007a). Therefore, the most important function of equine saliva appears to be the lubrication of swallowed ingesta and the buffering of gastric contents, which would promote intra-gastric enzymatic digestion and bacterial fermentation.

Adult horses may secrete up to 35-40 litres of saliva per day (pH of 8.6-9.1), which contains significant concentrations of bicarbonate (50 mEq/l) that functions as a buffer (Meyer *et al.*, 1985). The amount of saliva secreted increases with increasing dry matter (DM) content of the feed, due to the physical composition of the feed, and moreover, the time required for adequate mastication (Meyer *et al.*, 1985, 1986). Meyer and co-workers have shown that the DM content of swallowed material varies between 11-15% during intake of fresh or dried forage, and increases to 21-34% after intake of concentrates. Hay stimulates the highest amount of saliva production with a corresponding low %DM in the swallowed bolus, while pelleted compound feeds stimulate the least amount of saliva resulting in high %DM levels in the swallowed bolus (Table 2.2) (Meyer *et al.*, 1986). As in other animal species, the rate of saliva secretion is stimulated by food intake and mastication, and not by the sight or smell of feed or psychological stimuli as happens in superior mammals such as humans (Houpt, 2012, Frandson *et al.*, 2009).

Table 2.2. Saliva production for roughage and concentrate feeds.

Feed	Litres/ kg feed (as fed)	Litres/ kg feed (DM basis)	% DM of swallowed bolus
Grass	0.59	2.95	12.6
Grass/ alfalfa silage	2.35	4.49	15.2
Leaves	2.81	7.07	11.2
Hay	5.80	6.53	14.4
Straw	5.22	5.87	13.6
Pelleted compound feed	1.70	1.90	33.6
Sugarbeet pulp 90*	2.23	2.34	29.0
Sugarbeet pulp 49*	1.18	2.42	23.2
Sugarbeet pulp 35*	0.63	1.87	20.6

*Indicates the soaking-time (in minutes) for the sugarbeet pulp
DM – dry matter

Meyer *et al.*, (1986)

Monograstric stomach and chemical digestion

The masticated feed enters the stomach via the oesophagus, where acid digestion occurs. The horse has a simple monogastric stomach, similar in structure to dogs, cats, pigs and humans; but relatively smaller in comparison to its body size (Frandson *et al.*, 2009). The volume of the equine stomach, depending on the feed type eaten, is about 8% of the total GI tract volume of an adult horse (450-500 kg), and has a capacity of 8-15 litres (Figure 2.2). The small size of the stomach is a result of the evolution of horses as continuous grazers, designed to consume small frequent meals. This digestive adaptation allows a relatively quick flow of digesta through the stomach, as compared to ruminants (Janis, 1976, Stevens *et al.*, 1980). Most digesta is held in the stomach for a relatively short time, with the majority being passed to the duodenum within 60-120 minutes following a meal. However, due to the continuous feeding behaviour of feral horses, the stomach is rarely empty (Frape, 2010).

The equine stomach is divided into three regions, the cardiac, fundic and pyloric regions, extending from the terminal end of the oesophagus to the proximal end of the duodenum. As in most mammalian species, the major secretory product in the stomach of the horse is hydrochloric acid (HCl; gastric acid), secreted by the parietal cells. In contrast to carnivores, but similar to omnivores, horses secrete gastric acid continuously, even when the stomach is empty during spells of fasting (termed basal secretion) (Campbell-Thompson and Merritt, 1987). Healthy horses secrete approximately 200 µEq/kg/h of gastric acid, which is mixed with varying amounts of pancreatic and duodenal fluid that is refluxed into the stomach from the upper small intestine (Campbell-Thompson and Merritt, 1990, Kitchen *et al.*, 2000). Pepsin and lipase are digestive enzymes secreted by zymogen cells found in the fundic and pyloric regions of the stomach.

The glandular regions of the stomach produce mucus and bicarbonate that protect the surfaces from erosion by gastric acid. The meal size and composition of ingested feed (grain or forage) determine the amount of gastric acid secreted into the stomach lumen. Following a large grain meal, the secretion of gastric acid causes a rapid rise in pH, partly as a consequence of a delay in the secretion of gastrin (a peptide hormone that stimulates the secretion of gastric acid) compared with the rapid gastrin response to a forage diet such as hay (Frape, 2010). Therefore, a decrease in roughage intake may result in a drop in the mean pH in the upper part of the stomach $\leq$4.0, thereby exerting a challenge on the non-glandular part of the stomach which may ulcerate due to acid damage (Husted *et al.*, 2009). Furthermore, food entering the

stomach region may be poorly mixed, resulting in a pH gradient between the cardiac (pH 5.4) and pyloric (pH 1.8 - 2.6) regions (Al Jassim and Andrews, 2009). This variation occurs because roughage is of a lower density (larger particle size) than grain, and hence, roughage tends to float on the top of the digesta, whereby it is subjected to minimal exposure to the gastric acid (pH 2.0-3.0) produced in the lower glandular part of the stomach and maximal exposure to swallowed saliva (pH ~7.5) (Stick *et al.*, 1981).

Intra-gastric fermentation by microbial activity has been reported in the horse with bacteria in close association with the mucosa throughout the stomach. The abundance and diversity of mucosal microbiota in the stomach varies by individual and the type of diet consumed (de Fombelle *et al.*, 2003, Perkins *et al.*, 2012). More than 100 bacteria attached per epithelial cell have been reported, with the highest concentrations of lactate-utilising bacteria and *Streptococcus* spp. present in the stomach compared to other parts of the GI tract (de Fombelle *et al.*, 2003, Perkins *et al.*, 2012). Fermentation of non-structural carbohydrates by epiphytic microbiota (microbial species present on the surface of plant material) occurs in the oesophageal and fundic regions of the stomach, especially within the *Saccus ceacus*, yielding lactic acid and moderate concentrations of VFAs (also termed as short chain fatty acids) (Al Jassim *et al.*, 2005). However, despite large densities of microbial communities in the stomach, fermentative activity is weak, which is evident by the low concentrations of fermentation end-products such as acetate, propionate and butyrate (Varloud *et al.*, 2007a).

The small intestine and enzymatic digestion

Stomach contractions initiated by newly arrived feed, moves digesta further down the GI tract into the small intestine, where enzymatic digestion of protein, starch and other simple carbohydrates takes place (Van Weyenberg *et al.*, 2006, Julliand *et al.*, 2008). Relative to its size, the horse has a short small intestine measuring ~21-25 m in length in a 450-500 kg horse (Figure 2.2), as compared to that of ruminants (~40 m in cattle) (Nickel *et al.*, 1973). The small intestine constitutes 30% of the volume of the equine GI tract, and is a continuous segment (without junctions or valves) divided into three major portions - the duodenum, jejunum, and ileum.

The duodenum is the first portion of the small intestine, entered via the pyloric sphincter of the stomach, where neutralisation of the acidic pH occurs and enzymatic digestion begins. The horse lacks a gall bladder, and hence, bile is secreted directly into the duodenum

along with pancreatic secretions via a common duct located distal to the pylorus. The jejunum is the middle section of the small intestine. It is the longest portion of the small intestine, anatomically characterised by folded loops of intestinal lumen. Following the jejunum is a short ileum that is characterised by the presence of numerous goblet (mucous) cells and aggregates of lymphoid tissue (Peyer's Patches) towards the distal end. The lymphoid tissue produces protective cells (e.g. B-lymphocytes, T-lymphocytes, CD4+, CD8+ and CD25+ cells) that prevent potentially harmful microbes from the hindgut from colonising the small intestine. The ileum culminates at the ileo-caecal junction (Dyce *et al.*, 2009, Frandson *et al.*, 2009).

The lumen of the entire length of small intestine is lined by columnar epithelium to form numerous, densely packed villi and microvilli that contain brush border enzymes, which are responsible for the breakdown of disaccharides and the absorption of monosaccharaides and fatty acids. Horses also harbour anaerobic bacteria in the small intestine that aid in the process of digestion. For example, in grass-fed horses, there are about 2.9 x 10^6 CFU /g of bacteria in duodenum, 2.9 x 10^7 CFU /g in the jejunum and 3.8 x 10^7 CFU /g in ileum (de Fombelle *et al.*, 2003).

The small intestine is the major site for enzymatic digestion of the non-structural carbohydrate portion of the diet, and absorption of nutrients, including amino acids from proteins, simple sugars from carbohydrates, fatty acids from lipids, and some vitamins and minerals. Carbohydrates, some proteins, and fats are broken down and absorbed directly in the duodenum, while proteins are readily broken down to amino acids by proteolytic enzymes in the ileum. The release of acidic chyme (pH 2.5 to 3.5) from the stomach into the duodenum stimulates pancreatic secretions, the amount of which increases 3-4 fold within 2-3 minutes of the horse starting to eat (Sjaastad *et al.*, 2010). In comparison with other herbivores, horses produce large amounts of pancreatic juice (10 to 12 l/100 kg of body weight in a 24-hour period) continuously, which compares with 1-5 l/100 kg for sheep and cattle (Sjaastad *et al.*, 2010). The acidic bolus of food from the stomach is neutralised by bicarbonate secreted from the Brunner's glands, bile salts from the liver via the bile duct, and digestive enzymes such as trypsin, lipase and amylase from the pancreas. The action of bile buffers the pH to approximately 7.0-7.5, while the duodenal glands (Brunner's glands) secrete bicarbonate (HCO_3^-) neutralising the acidic chyme, and providing an optimal pH for the action of pancreatic enzymes. The pH in the jejunum and ileum ranges from neutral (7.0) to slightly alkaline (7.8-8.2), which is essential for the active transport of nutrient substrates across the

intestinal mucosa and optimal activity of the enzyme systems such as amylases and lipases. Bile salts cause the emulsification of fat into smaller globules, which increase the surface area exposed for enzyme interaction. The fat is then hydrolysed by lipase enzymes (from the pancreas) to produce micelles of fatty acids and glycerol, which are absorbed through the small intestinal epithelium (Sjaastad *et al.*, 2010).

Digestion of starch in the small intestine of the horse is inefficient, because the horse produces only 8-10% of the amylase normally produced in other species such as the pig (Jackson, 1998). Utilisation of starch is mostly the result of degradation by pancreatic amylases, brush border disaccharidases and limited microbial digestion. The starch degraded by α-amylase releases maltose, maltotroise and α-dextrin, which is then hydrolysed to yield glucose (Frandson *et al.*, 2009). The glucose released is then transported across the small intestinal epithelium by Na^+ dependent (active transport) and Na^+ independent (facilitative diffusion) transport mechanisms. Except for residual starch, most of the available nutrients are absorbed by the time the digesta reaches the terminal ileum. Substrates that have not been absorbed in the small intestine are carried forward into the large intestine with the passage of digesta (Julliand *et al.*, 2006).

Feed intake levels influence the passage rate of digesta through the small intestine (Van Weyenberg *et al.*, 2006). Large amounts of feed ingested in a meal, increases the rate of outflow from the small intestine into the large intestine, with 50% of the digesta found in the distal ileum at 60 minutes and 25% in the caecum at 90 minutes (some present within 45 minutes) post-ingestion (Merritt, 1999, Frape, 2010). The physical form of feed also influences the passage rate. Pelleted rations transit quicker than textured grain mixes combined with hay, while liquids may reach the caecum a few hours after ingestion (Al Jassim and Andrews, 2009). Additionally, due to the differences in the DM content of roughage, the passage rate of dietary fibre may alter the viscosity and water-holding capacity of the digesta, thereby altering the dynamics of flow and the uptake of nutrients in the small intestine (Drogoul *et al.*, 2000b), before entering into the large intestine.

2.2.2.2 Hindgut anatomy and microbial fermentation

Anatomy of the caecum, colon and rectum

The hindgut (large intestine) comprises the caecum, large and small colon, and the rectum, and constitutes the major proportion (~50-60%, ~95-112 L capacity) of the equine GI

tract (Figure 2.2). In the adult 450-500 kg horse, the average caecal volume is 33 L and that of the large colon is 80 L, which accounts for ~60% of the total GI tract volume (Nickel *et al.*, 1973). The caecum and large colon are the major sites for digestion of structural carbohydrates (such as cellulose and hemicellulose) via microbial fermentation to produce VFAs that are utilised to meet energy requirements (Frandson *et al.*, 2009).

Anatomically, the caecum is a large, blind, comma-shaped structure (~1 m length in a 450-500 kg horse) (Nickel *et al.*, 1973). Unlike other herbivores, the small intestine in the horse opens directly into the caecum through a muscular valve located at the ileo-caecal junction (ileal orifice). A second valve (caeco-colic orifice) controls the passage rate of digesta from the caecum into the right ventral colon. The inlet and outlet of the caecum are positioned in close proximity to each other, forming a 'T' shaped structure (Dyce *et al.*, 2009). Under the influence of muscular contractions of the caecum, the digesta is effectively mixed, undergoes fermentation, and are eventually passed through the caeco-colic opening into the colon.

The colon in the horse (~10 m in length in a 450-500kg horse) is divided into several parts, based on the anatomic position. The ascending colon of the equine hindgut (large or greater colon) is highly modified and capacious, and is anatomically compartmentalised into four sections - right ventral colon, left ventral colon, left dorsal colon and right dorsal colon (Figure 2.2). These compartments are folded in the abdominal cavity, to form a double horse-shoe-shaped loop and the sternal, pelvic and diaphragmatic flexures (Dyce *et al.*, 2009). The ventral colon can hold a volume of ~150 L, has a diameter of 250 to 300 mm, and is ~2-4 m long. The diameter varies significantly between various regions of the colon, and is largest at the right dorsal colon. The passage between the left dorsal colon and right dorsal colon is relatively narrow at the pelvic flexure, resulting in delayed transit of large particles from the more proximal compartments (e.g. from the left ventral colon to the left dorsal colon). The ascending colon is followed by a short narrow transverse colon (diameter ~10 cm) and the descending colon (small or lesser colon, length 3.5 m), which joins the dorsal colon to the rectum in the pelvic cavity and ends at the anus (Dyce *et al.*, 2009, Frandson *et al.*, 2009).

The walls of the large intestine contain numerous glands (crypts of Lieberkuhn) which secrete mucus throughout the lumen of the large intestine. Villi are absent, but microvilli that do not produce digestive enzymes are present on the apical surface of the columnar cells that line the luminal surface of the caecum and colon. The continuous production of pancreatic juice and bile in the small intestine ensures a steady flow of buffered

intestinal contents to the large intestine, and the high water content of the pancreatic juice ensures favourable conditions for the maintenance of a diverse microbial population in the caecum and colon. Distinct bands of muscle (taenia) are present along the length of the large intestine, which gather the walls into sacculations called haustra (Dyce *et al.*, 2009). The sacculations allow for greater digesta holding capacity and prolongs the transition of digesta through the large intestine. These anatomic structures of the hindgut also increase the retention time of digesta for fermentation by microbes, and allow greater surface area for absorption of VFA, electrolytes and water in throughout the large intestine (Hintz and Cymbaluk, 1994).

Microbial fermentation in the equine hindgut

The mammalian gut is incapable of producing enzymes that digest cellulose (Janis, 1976), so in order to digest fibrous plant material, a diverse population of symbiotic microorganisms inhabits the equine hindgut and play a major role in fibre digestion. This symbiotic relationship enables horses to utilise structural carbohydrates as the primary source of metabolisable energy (Clauss *et al.*, 2003). The diverse community of hindgut microbiota are comprised of bacteria, archaea, fungi, ciliate protozoa, phages and other unknown microorganisms. Two functions are attributed to the hindgut microbiota: 1) the digestion and metabolism of carbohydrates that are undigested in the small intestine (cellulose and residual starch), and 2) the synthesis of vitamin K and many B-vitamins (Dicks *et al.*, 2014).

Microbial fermentation in the hindgut occurs under anaerobic conditions (Hintz *et al.*, 1978). Bacteria, fungi, and some cellulase-producing ciliate species of microorganisms, break down the cellulose polymers and ferment the resulting monomers and oligomers to produce VFAs, which are the main energy source for the horse. The principle VFAs produced in the hindgut are acetate, propionate and butyrate. The ratio of the VFAs produced varies with diet, although acetate is the major product. When horses are fed a high fibre diet, the molar ratio of acetate to propionate to butyric acid produced is approximately 70:20:10 (Hintz *et al.*, 1978, Stevens *et al.*, 1980). Other end-products of microbial fermentation include lactic acid, methane, hydrogen and carbon dioxide (Hintz *et al.*, 1978). Microbes do not digest dietary fat. Residual carbohydrates (including starch, pectin and fructans), that are undigested in the small intestine are fermented in the caecum and colon. Lactic acid that is produced from microbial fermentation of starch in the small intestine is not well absorbed and is transported to the caecum where it is fermented to propionate (Frape, 2010). The VFAs produced in the hindgut are readily absorbed into the bloodstream through intestinal epithelial cells via diffusion, along

with water and electrolytes (Argenzio *et al.*, 1974). The pathways of carbohydrate fermentation are shown in Figure 2.3.

Proteolytic bacteria in the hindgut break down excess protein that is not digested in small intestine. Most of the essential amino acids absorbed by the horse are obtained from plant material, and only 1-2% of the amino acids are of microbial origin. The remaining microbial protein is voided in the faeces (Cunha, 1991). Some amino acids in the large intestine are decarboxylated to amines and deanimated to urea in the liver. Urea is then secreted into the ileum and transported to the caecum where bacteria hydrolyse it to ammonia, which is used in turn for protein synthesis by other bacterial species (Figure 2.3) (Stevens and Hume, 1998). Hydrogen produced via microbial fermentation serves as an electron donor in the energy metabolism of methanogenic archaea and is converted to methane gas (CH_4). The amount of methane produced depends on the microbial species and the relative size of the fermentation chamber. Cattle produce about 35-55 kg of CH_4, sheep and goats produce about 5-8 kg CH_4 and horses produce 18 kg CH_4 per year (Stevens *et al.*, 1960, Crutzen *et al.*, 1986).

Figure 2.3. Bacterial metabolism of nitrogen and fermentation of carbohydrates in hindgut.

Adapted from Stevens and Hume (1998)

During microbial fermentation, synthesis of vitamin K and many B-vitamins occurs. Considerable quantities of thiamine (B1), riboflavin (B2), nicotinic acid (B3), panthothenic acid (B4), pyridoxine (B6), biotin (B7) and folic acid (B9) are synthesised in the hindgut, with the greatest amounts produced in the caecum and colon (Carroll *et al.*, 1949). Vitamin K and B12 are synthesised in sufficient quantities to meet the requirements of horses. However, only ~25% of the B1 synthesised may be absorbed through the intestinal epithelium, while the remaining B-vitamins are excreted in the faeces and may only be available to the animal if re-ingested via coprophagia (Carroll *et al.*, 1949).

Importance of the intestinal microbiome

The intestinal (gut) microbiome is comprised of biotic (microbial communities), and abiotic (environmental elements such as nutrients, water and pH), components which interact with each other to maintain the digestive, metabolic and immune function of the host. The definition of the terms microbiome and microbiota are largely synonymous, and these terms are often used interchangeably. However, some scientific articles distinguish the two terms to

describe either the collective genomes of the microorganisms that reside in an environmental niche (microbiome) or the microorganisms themselves (microbiota) (Ley *et al.*, 2006, Turnbaugh *et al.*, 2007).

Within a given intestinal environment, some microbial members function as entrenched 'residents' (autochthonous components), while others act more like 'hitchhikers' (allochthonous members) arriving via ingested food, water, and various other components of the environment (Ley *et al.*, 2006). The populations of microbiota are thought to be stable (the abundance of microbial communities remains constant) within the gut environment, if not resilient (the abundance of microbial communities fluctuate within normal range) (Lozupone *et al.*, 2012). In horses, the hindgut microbiota have a symbiotic relationship with the host facilitating the digestion of fibre, while the host contributes a regular carbohydrate food source for the microbiota (Santos *et al.*, 2011).

Recent studies on the gut microbiota of horses have demonstrated dramatic changes in the microbiota in relation to diet, disease, or environmental factors (de Fombelle *et al.*, 2001, Kobayashi *et al.*, 2006, Milinovich *et al.*, 2006, Willing *et al.*, 2009, Costa and Weese, 2012). Furthermore, many important disorders in horses, including laminitis, equine metabolic syndrome, colic, gastric ulcers, antibiotic associated diarrhoea, are associated with disruptions in the gut microbiota. Therefore, there is great interest in furthering our understanding on the complexity of this intestinal microbiome (Venable *et al.*, 2016).

The maintenance of a healthy microbiota may be critical for optimal digestive function. Recent findings have suggested that a highly diverse, yet stable intestinal microbiome may play an important role in maintaining health and preventing the occurrence of disease (Costa and Weese, 2012, Lozupone *et al.*, 2012, Taschuk and Griebel, 2012). However, the complexity of the equine hindgut microbiome and the factors that affects its normal composition are still poorly understood (Costa and Weese, 2012).

2.3 Factors that affect the hindgut microbiome

The symbiotic microbes that are normal inhabitants of the equine hindgut are sensitive to changes in the GI tract environment (e.g. pH, availability of substrates and GI motility). These microbes require optimal conditions for effective fermentation activity to occur within the hindgut. Several factors affect the intestinal microbiome and cause disruptions to the gastrointestinal ecosystem, which in turn compromises digestion, health, and performance of the host.

2.3.1 Diet and feeding behaviour

The type and composition of the diet influences the hindgut microbiome and the digestibility of the feed (de Fombelle *et al.*, 2003). For example, fermentation of starch in the hindgut could affect the pH and the population of fibrolytic microbes. Excess levels of fat in the diet (by encapsulation) may decrease the pre-caecal digestion of starch, which may lead to rapid fermentation of starch in the hindgut causing a change in pH, and this may indirectly reduce the microbial fermentation and digestibility of fibre (Rossiter, 2008).

Roughages, such as hay and other conserved forages, are believed to play an essential role in buffering and preserving the digestive function of the hindgut microbiome (de Fombelle *et al.*, 2001). Horses fed 100% hay produced uniform proportions of lactic acid that decreased hindgut acidosis. Acidic faecal pH (pH $\leq$6.32) was associated with horses that were offered more concentrate feed (4 kg of grain), and $\leq$2.25 kg hay/day. If hay was reduced to <2.25 kg/day, a more acidic pH was observed than horses fed higher quantities of hay (Williamson *et al.*, 2007). This may be because hay has a high DM content (85-95%) that increases saliva secretion due to increased mastication, which buffers the pH in the hindgut. Secondly, forage favours a more favourable VFA production (higher acetate and lower propionate ratios), which positively influences digestive function and absorption of nutrients and energy (Frape, 2010).

As stated previously (section 2.1.2), intensively managed athletic horses are fed energy-dense diets, and these diets are consumed relative quickly, which reduces the feeding time compared to horses fed a diet high in fibre (Ellis and Hill, 2005, Richards *et al.*, 2006). Ponies are efficient feed converters, and when pasture access is restricted to lower caloric intake, these ponies increase their voluntary feed intake to compensate for the energy deficit (Dugdale *et al.*, 2010, Glunk *et al.*, 2013a). Accelerated feed intake could potentially lead to

an increased amount of fermentable substrate (non-structural carbohydrates) reaching the hindgut, leading to enhanced fermentation and reduced hindgut pH (Milinovich *et al.*, 2008).

Evidence suggests that the population of hindgut microbiota are highly sensitive to dietary changes (such as increased hydrolysable carbohydrates and reduced roughage), which result in a significant decrease in caecal and colonic pH (Julliand *et al.*, 2001, Medina *et al.*, 2002), and perturbation in the microbiota. Disturbances in the microbial populations place the horse at a greater risk of developing intestinal dysfunction (Goodson *et al.*, 1988, Clarke *et al.*, 1990). Therefore, maintaining a consistent balance in the microbial population of the equine hindgut by feeding a consistent diet has been suggested (Sadet-Bourgeteau and Julliand, 2010).

2.3.2 Passage rate of digesta

The passage rate of digesta is the time taken for digesta to travel through the GI tract from ingestion to excretion (Van Weyenberg *et al.*, 2006). The digestibility of ingested feed is influenced by the composition of microbial communities, and the length of time feeds are retained in the digestive tract (Koller *et al.*, 1978). The longer the feed is retained at the site of digestion, the greater the digestibility, because fibre is exposed to microbial activity for a longer time (Mertens and Ely, 1982). However, due to the post-gastric placement of the fermentation chamber in the horse, utilisation of protein from microbial fermentation is less efficient when compared to the ruminant. In order to meet their protein and energy needs, feed intake in horses is higher than ruminants, which in turn increases the passage rate of digesta and decreases the fermentation time (Bell, 1971).

In stabled horses fed grain and forage, the transit time of a large meal through the stomach may be shorter than that of feed eaten continuously at lower volumes. The passage rate through the GI tract may be influenced by several factors including the type and volume of feed ingested, and individual variation between animals (Pearson *et al.*, 2006, Van Weyenberg *et al.*, 2006, Al Jassim and Andrews, 2009). Research on the digestive physiology of horses has repeatedly shown that, as in any herbivore, variation in food intake leads to a variation in both digesta retention (higher intakes are linked with shorter retention times) and digestibility (shorter retention times are usually linked with lower digestibility) (Pearson *et al.*, 2001, Ragnarsson and Lindberg, 2010). However, in horses, the gain in digestible nutrients with increasing intake usually exceeds the disadvantage of reduced digestibility.

2.3.2.1 Markers used to assess GI passage rate

Measuring the passage rate of digesta through the GI tract can provide indirect information on the extent of digestion and fermentation of feed, and this can be measured by using indigestible markers such as dyes, solid particles or rare-earth molecules attached to the feed particles or mixed loosely in the diet. A marker is selected depending on the diet studied and is fed to the horse, and digesta or faecal samples are collected at set sampling points for the analysis of the amount or concentration of the marker excreted in the faeces (Sales, 2012).

Digesta passage through the GI tract can be divided into two phases, particulate (solid) and solute (liquid). The coordinated retropulsive-propulsive (to and fro) movements of the caecum and colon cause a physical separation of the finer particles from the less digested coarse particles (longer than 1 cm), the latter are pushed back into the ventral colon (Sellers and Lowe, 1986). Differential movement of fluid and particulate digesta through the gut warrants the use of different markers for each phase (Stevens and Hume, 1996).

Markers used to investigate the passage rate must have the following characteristics: a) be inert or non-toxic, b) be neither absorbed nor metabolized in the GI tract, c) must have no appreciable bulk, d) mix and remain uniformly distributed in the digesta, e) must not affect the GI tract and microbial population, and f) must be quantifiable (Kotb and Luckey, 1972). Examples of markers used for evaluation of the fluid phase include ^{51}Cr-EDTA and Co-EDTA (Cuddeford *et al.*, 1995), while unlabelled ruthenium-phenanthroline (Ru-P) (Orton *et al.*, 1985), Cr-mordanted hay fibre (Cr-fibre) (Udén *et al.*, 1982), styrofoam particles (Hintz and Loy, 1966), coloured beads (Wolter *et al.*, 1974) and rare earth labelled fibres (Dysprosium (Dy), Europium (Eu) or Ytterbium (Yb)) (Todd *et al.*, 1995, Drogoul *et al.*, 2001) have been used for evaluation of the passage of the particulate phase (Table 2.3).

Most previous studies have used solid phase markers to investigate changes in the mean retention time (MRT) of feeds in the digestive tract of horses and ponies under different management conditions (McGreevy *et al.*, 2001, Pearson *et al.*, 2001, Boscan *et al.*, 2006, Pearson *et al.*, 2006). However, because most previous studies investigating the passage rate of digesta have used different markers fed with different types of feeds, making comparisons between the results of these studies is challenging.

A two compartment model (using two types of markers) has been used to study intestinal motility in ruminants and horses, and the findings indicate that significant

differences existed between the MRT of solid and liquid phase markers (Udén *et al.*, 1982, Rosenfeld *et al.*, 2006). Liquid MRT is typically shorter than particulate MRT, therefore the average MRT of the solid and liquid phase markers is often used to determine total gastric transit or mean retention times (Dhanoa *et al.*, 1985). In horses fed timothy hay, only small differences in MRT were apparent between the solid and liquid phase markers, possibly due to the anatomical placement and motility of the hindgut compared to the rumen (Udén *et al.*, 1982). This indicated that measuring the solid phase might be more accurate in horses compared to ruminants. However, large individual variations in the passage of solid and liquid phase markers were observed between horses fed on the timothy hay, which were not related to the size of the animal (Udén *et al.*, 1982).

2.3.2.2 Factors that affect passage rate

In the horse, most digesta is held in the stomach for a limited time (~30-120 min), following which the digesta transits rapidly (~30 cm/ min) through the small intestine via peristaltic movements. Within 3 h of feed consumption, the digesta reaches the caecum and right ventral colon where it is retained for variable lengths of time (Van Weyenberg *et al.*, 2006). The passage rate of the digesta influences the type of substrate that arrives in the hindgut, which is dependent on the time available for pre-caecal digestion.

Several animal- and feed-related factors affect the passage rate of digesta, such as animal weight, pregnancy, lactation, exercise, type of diet and feeding level (Van Weyenberg *et al.*, 2006). For example, the MRT of digesta was significantly lower in light breed horses compared to heavy breed horses, regardless of the feeding level (Miraglia *et al.*, 1992). The transit time in ponies is thought to be quicker than that of horses, but it could vary depending on the voluntary feed intake of the animal (Cuddeford *et al.*, 1995). Some studies have reported a large individual variation in nutrient digestibility with no significant differences between MRT and body size (Udén and Van Soest, 1982, Drogoul *et al.*, 2001).

When forage intake was restricted to ~70% *ad libitum* intake, the MRT of the solid phase increased by 12-36% and liquid MRT increased by 11 – 21% (Pearson *et al.*, 2001, 2006). The particulate and liquid MRT decreased when ponies were allowed *ad libitum* access to grass hay (93 g/kg $BW^{0.75}$) compared to being fed restricted amounts (77 g/kg $BW^{0.75}$, 55 g/kg $BW^{0.75}$, and 31 g/kg $BW^{0.75}$) (Clauss *et al.*, 2014). Consumption of long-stemmed hay increased the rate of passage through the hindgut, compared with ground and pelleted hay diets with a much smaller particle size (Drogoul *et al.*, 2000a). The smaller particles of the pelleted

diet were retained longer in the large colon (92% of the MRT) than the bigger particles (80% of the MRT). Feeding pelleted diets not only slowed down the passage rate, but also led to similar passage rates of the solid and liquid phases, due to the formation of a more homogenous digesta (Drogoul *et al.*, 2000a).

The solid phase MRT of the digesta reported in horses fed alfalfa hay was 30 h, whereas the oat straw MRT was 36 h when horses were fed at similar intake levels (Pearson *et al.,* 2001). Moore-Colyer *et al.,* (2003) showed that a low-fibre diet (NDF = 50%) had an increased MRT (28 h) compared to a high-fibre diet (NDF = 70%, MRT = 24 h) fed at similar intakes. Furthermore, when different chopped lengths (29.5 cm and 6.8 cm) of silage (fermented in wrapped bales and chopped before feeding) were compared, shorter MRTs were associated with the shorter fibre length (Moore-Colyer *et al.,* 2003). No significant differences were observed between the transit times of hay and alfalfa, but increasing the proportion of alfalfa increased passage rate, perhaps due to the reduced fibre length of the alfalfa (2–4 cm) compared to the hay used in the study (Cuddeford *et al.*, 1992). The variation (14-60 h) in the MRT of digesta observed between different studies is shown in Table 2.3.

Table 2.3. Mean retention time (MRT) (h) of digesta in horses (H) and ponies (P) fed various diets and measured with different markers.

Reference	Animal	Diet	Marker	MRT (h)
Hintz and Loy (1966)	H	Barley grain	Styrofoam particles	25 (pelleted) 33 (unpelleted)
Wolter *et al*., (1974)	P	Meadow hay	Coloured beads	36
		Chopped meadow hay	Coloured beads	25
		Pelleted meadow hay	Coloured beads	29
Wolter *et al*., (1976)	P	Alfalfa/grain	Coloured beads	29
Udén *et al*., (1982)	H,P	Timothy hay	Co-EDTA	18
			Cr-mordanted fibre	23
Orton *et al*., (1985)	H	Chopped oaten	Cr-EDTA	22
		Hay/concentrates	Ru-P	27
Pearson and Merrit (1991)	P	Meadow hay	Co-EDTA	31.3
			Cr-fibre	29.9
		Barley straw	Co-EDTA	34.1
			Cr-fibre	34.8
Miraglia *et al*., (1992)	H	Hay/concentrates	Cr-mordanted fibre	25.2–36.5[a]
Cuddeford *et al*., (1995)	H,P	Alfalfa	Co-EDTA	53.5
			Cr-fibre	59.6
		Oat straw	Co-EDTA	45.6
			Cr-fibre	47.6
Todd *et al*., (1995)	H	Alfalfa cubes/vitamin pellets	Dy	14.1–24.6–25.8[b]
Pagan *et al*., (1998)	H	Alfalfa cubes + alfalfa grass hay	$YbCl_3 . 6 H_2O$	34
		Grain + Alfalfa cubes		43.4
Drogoul *et al*., (2001)	P	Chopped hay/rolled barley	Eu (for hay)	30.1–35.4–42.2[c]
			Yb (for barley)	35.7–42.9[c]
			Cr-EDTA	34.9–37.6–44.5[c]
Pearson *et al*., (2001)	P	Alfalfa	Co-EDTA	20.5
			Cr-fibre	21.3
		Oat straw	Co-EDTA	30
			Cr-fibre	31.5

Legend: [a] Depending on animal weight; [b] Depending on the method of calculating MRT; [c] dependent on the ratio of hay/barley. Cr – chromium, Co – cobalt, EDTA – ethylene diamine tetra acetic acid, Ru-P - ruthenium-phenanthroline, Dy – dysprosium, $YbCl_3 . 6 H_2O$ - ytterbium chloride, Eu – europium, Yb – ytterbium.

Adapted from Van Weyenberg *et al*., (2006)

2.3.3 Hindgut and faecal pH

Intestinal pH in the horse varies depending on the segment of GI tract and the diet consumed (Merritt and Julliand, 2013). Significant changes in hindgut pH occur throughout the day, with a rapid decrease in pH during the hours after feeding that reaches a minimum 5 h post-feeding. The pH remains at this low level for 2-4 h, after which it increases progressively until the next meal (Goodson *et al.*, 1988). The rate and extent of the pH drop differs depending on the diet, and the decrease is more pronounced with a diet rich in concentrate than with a diet rich in forage (Merritt and Julliand, 2013).

Many studies have investigated the effects of diet on the hindgut and faecal pH (de Fombelle *et al.*, 2003, Berg *et al.*, 2005, Müller *et al.*, 2008). Reliable data on hindgut pH can be obtained using caecal cannulation (Julliand *et al.*, 2001, Medina *et al.*, 2002), however, data on faecal pH appears unreliable due to the large variation and inconsistencies observed between studies (Berg *et al.*, 2005, van den Berg *et al.*, 2013, Kristoffersen, 2014). This variation may be due to differences in the dietary treatments, type of animals used, site of pH measurement or limitations in detecting subtle changes in the faecal pH, especially when forage-based diets are fed. Nevertheless, changes in pH are associated with a number of digestive-related disorders such as gastric ulcers, hindgut acidosis, colitis, colic and laminitis (Al Jassim and Andrews, 2009). Therefore, maintaining an ideal pH in the GI environment is an important factor for proper function of the digestive tract, mainly because the pH influences the microbial diversity and the digestibility of feed in the hindgut (Zeyner *et al.*, 2004).

2.3.4 Microbial communities (Microbiota) in the hindgut and faeces

In horses, few studies have identified and characterised the microbial species in the stomach and small intestine (Al Jassim and Andrews, 2009, St-Pierre *et al.*, 2013), and most studies over the last decade have focused on the larger proportion of gut microbiota that inhabit the horse's hindgut, including faeces (Costa and Weese, 2012, Dougal *et al.*, 2012, Dougal *et al.*, 2013).

2.3.4.1 Quantifying the intestinal microbiota

Historically, the equine hindgut and faecal microbiota were studied using culture-dependent techniques. Samples obtained from the hindgut or faeces were collected, grown on the nutrient media under anaerobic incubation in the laboratory, and the number of live

organisms was counted. The microbial isolates were then identified by using biochemical methods, to provide an overall, non-invasive assessment of the microbial population in the hindgut or faeces (Moore and Dehority, 1993, de Fombelle *et al.*, 2003, Respondek *et al.*, 2008, Muhonen *et al.*, 2010, van den Berg *et al.*, 2013). However, only 10-15% of the intestinal microbiota were able to be cultivated by standard culture techniques, due to the strict anaerobic growth requirements of the majority of the microbial species (Zoetendal *et al.*, 2004). The high diversity of microbial species and difficulties with culturing fastidious anaerobic organisms (organisms that require specific nutrient requirements) made it challenging to assess or compare microbial populations in the equine hindgut.

Estimates of total microbial counts in the colon and caecum of horses were compared from previous culture-based investigations (Costa and Weese, 2012)(Table 2.4). These values were found to be many log-units (CFU) less than the values reported by an early study that used quantitative real-time polymerase chain reaction (qPCR) (Milinovich *et al.*, 2006), a culture-independent technique. This finding demonstrated a major weakness in the culture-based data, namely that culturing a sample only detected a small fraction of the total microbial population. Nonetheless, culture-based techniques typically provided a superficial overview of the bacterial populations, and although changes detected from culture-based techniques could indicate significant changes in the population of microbiota, caution was warranted with interpretation of this data (Costa and Weese, 2012). Furthermore, there was increasing interest in investigating the complete spectrum of the hindgut microbiota (bacteria and other microbes), and hence the use of culture-independent molecular techniques became essential (Costa and Weese, 2012).

Table 2.4. Bacterial population of the equine intestinal tract estimated by culture and qPCR techniques.

Method	Organism(s)	Site	Log_{10} CFU/g	Reference
Culture	Total anaerobes	Colon	7.7	Muhonen *et al*., (2009)
	Total anaerobes	Colon	6.8-7.4	Respondek *et al.,* (2008)
	Total anaerobes	Stomach	4.8-8.8	Varloud *et al.,* (2007a)
	Total anaerobes	Faeces	8.6	Garrett *et al.,* (2002)
	Total bacteria	Stomach	9.1	de Fombelle *et al.,* (2003)
	Total bacteria	Cecum	7.7	de Fombelle *et al.,* (2003)
	Total bacteria	Faeces	8.5	de Fombelle *et al.,* (2003)
qPCR	Total bacteria	Cecum	11.0	Millinovich *et al.,* (2006)

Adapted from Costa and Weese (2012)

Metagenomics refers to culture-independent studies of the collective set of genomes of mixed microbial communities and applies to explorations of all microbial genomes that reside in environmental niches, in plants, or in animal hosts (Petrosino *et al.,* 2009). This science of biological diversity combines the use of molecular biology and genetics to identify and characterise genetic material from complex microbial environments (Allen and Banfield, 2005). The combination of metagenomics and subsequent quantitation of each identified species using molecular techniques allows the relatively rapid analysis of the microbiome (Furrie, 2006).

Two types of DNA-based microbiome studies are used; targeted amplicon studies that focus on one or a few marker genes and use these markers to reveal the composition and diversity of the microbiota (such as 16S rRNA gene amplicon sequencing), or studies that use an entire metagenomic approach (such as shotgun metagenomics) (Kuczynski *et al.*, 2012a). The 16S rRNA gene is a section of the prokaryotic DNA found in all bacteria and archaea, which codes for the smaller subunit of the ribosomal unit. The 18S rRNA gene is a homologous marker gene in eukaryotes that can be amplified to identify the protozoa and fungal species (DeSantis *et al.*, 2006).

Prior to sequencing, molecular techniques utilised PCR-based amplification of the 16S rRNA gene, followed by visual comparison of the amplicons using methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and fluorescent *in situ* hybridisation (FISH). Other methods of characterising the marker genes include terminal-restriction fragment length polymorphism (T-TFLP) analysis,

single strand conformation polymorphism (SSCP) analysis, dot-blot hybridisation, quantitative real-time PCR (qPCR) and a range of microarrays (O'Sullivan, 2000, Zoetendal *et al.*, 2004). All of these molecular methods are useful when comparing limited numbers of microbial communities in small set of samples.

When comparing large number of samples obtained from environments containing highly diverse microbial communities, it is impractical and uneconomical to use the above-mentioned techniques. The current most frequently used high-throughput molecular techniques include 16S and non-16S rRNA amplicon generation using PCR based amplification, followed by capillary (Sanger) sequencing (such as the Applied Biosystems DNA analyser), or next-generation sequencing on the more recently developed platforms (Illumina and 454-pyrosequencing), which allow the use of universal primers to cover the diversity of microbes in the samples obtained from the gut environments (Kuczynski *et al.*, 2012a).

Most of the molecular techniques used in investigations of the equine hindgut microbiome have been adapted from work carried out in the human microbiome (Turnbaugh *et al.*, 2007). Currently, there appears to be no 'gold standard' technique for the assessment hindgut microbiota. Over the past decade, several molecular techniques have been used to identify and characterise the microbiota in the equine gastrointestinal tract (stomach and large intestine), and to investigate changes in the populations of microbiota in relation to diet, disease, management or environmental factors (Costa *et al.*, 2012, Faubladier *et al.*, 2013, O' Donnell *et al.*, 2013, Dougal *et al.*, 2014). While metagenomics-based techniques have their advantages, in analysing different aspects of the intestinal microbiome, some limitations persist.

For example, 16S rRNA gene sequence informatics is useful for taxonomic classification of prokaryotic organisms, where phylogenetic analysis is performed by comparing the highly-conserved regions within the 16S rRNA gene sequence (Daly *et al.*, 2001, Shepherd *et al.*, 2012). Nonetheless, 16S rRNA gene sequencing provides genus identification in most cases (>90%), but is less accurate with regard to species (65-83%), with 1-14% of the isolates remaining unidentified (Lee *et al.*, 2012). Difficulties encountered in obtaining genus and species identification include the recognition of novel taxa, limited sequences deposited in nucleotide databases, species sharing similar and/or identical 16S

rRNA sequences, or nomenclature inconsistencies arising from multiple sequences assigned to single species or complexes (Woo *et al.*, 2008, Lee *et al.*, 2012).

Other limitations with assessing the microbiota of the hindgut include factors such as variability between gut regions (faeces versus caecum and colon), and variability due to study protocols such as differences in the study (horse) populations, diets fed to the animals, management, and also differences in the phylogenetic or statistical analyses (Costa and Weese, 2012). Furthermore, in order to make meaningful comparisons between and within subjects, most investigations require a large number of samples to be analysed, which can be expensive and time consuming (e.g. by DGGE analysis) (Earing *et al.*, 2012, Li *et al.*, 2012). Comparative metagenomics can be a useful technique to investigate whole-community microbiome diversity and functions (Turnbaugh *et al.*, 2007). However, the proper use of metagenomics in equine hindgut investigations requires further improvement with regard to optimal testing protocols, the relationship of faecal microbiota to more proximal locations in the hindgut (e.g. the caecum and colon), where disease is most likely to occur, normal intra- and inter-horse variation in different age populations managed under various conditions, and seasonal and geographical variations in the microbiota (Costa and Weese, 2012).

Next-generation sequencing techniques

Improvements in the analysis of highly diverse microbiomes has been achieved by using multiplexed barcoded PCR followed by high-throughput sequencing using next-generation sequencing technologies (Schuster, 2007). Examples of next-generation DNA sequencing platforms currently available include 454 pyrosequencing, Illumina (Solexa) sequencing, SOLiD and HeliScope (Ansorge, 2009). The two high-throughput sequencing platforms most commonly used to investigate the equine hindgut microbiome are 454 pyrosequencing (454 Life Sciences, Roche Applied Sciences, Basel, Switzerland) and Illumina (Solexa) sequencing (Illumina, San Diego, CA), and both these methods use the approach of ‘sequencing by synthesis’.

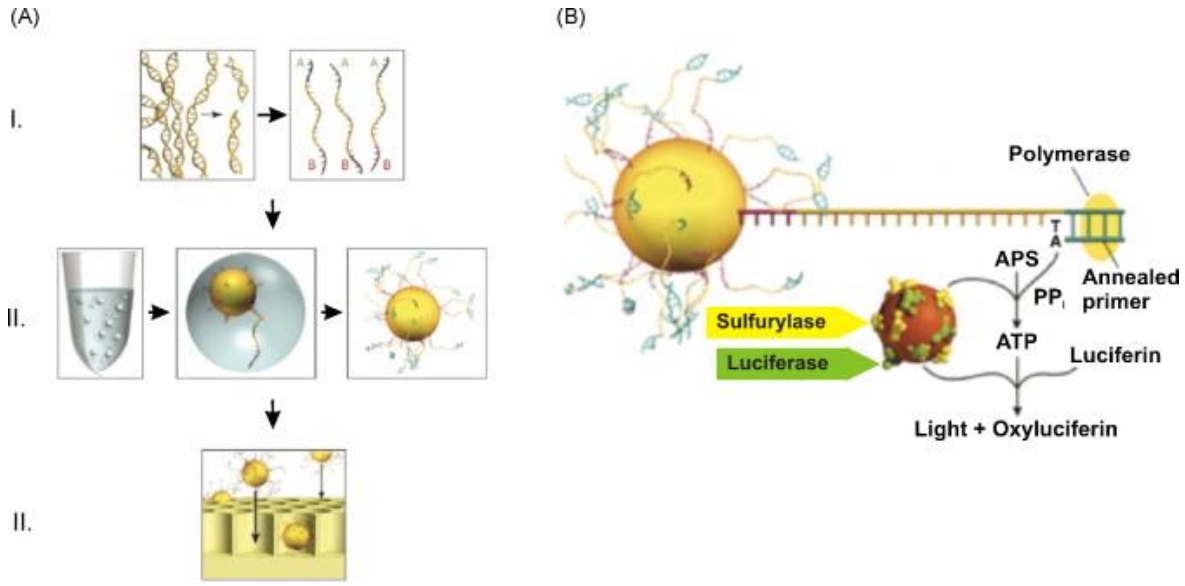
GS FLX 454 pyrosequencing

In this system, DNA fragments are ligated with specific adapters that cause the binding of one DNA fragment to a bead (Figure 2.4). The amplification is referred to as ‘sequencing-by-synthesis’, where emulsion PCR (emPCR) is carried out for amplification of the DNA fragments, with water droplets containing one bead and PCR reagents immersed in

oil. When the emPCR amplification cycles are completed, each bead with its one amplified fragment is placed in an optical fibre chip, after which polymerase enzyme and primers are added to it. To commence synthesis of the complementary strand, one unlabelled nucleotide is supplied to the reaction mixture in every bead on the chip. Incorporation of a following base by the polymerase enzyme in the growing DNA strand releases a pyrophosphate group, which can be detected as emitted light, which determines the identity of the nucleotide supplied in each step (Ansorge, 2009).

The 454 pyrosequencing method provides sequences with reading length of 400–500 bases with paired-end reads. However, certain drawbacks of this method, such as the relatively high cost of operation and high errors in sequencing of homopolymers (Schuster, 2007), led to the discontinuation of 454 pyrosequencing services by Roche Applied Sciences in 2013.

Figure 2.4. Schematic diagram of the 454 pyrosequencing platform.



Legend: (A) Outline of the GS 454 DNA sequencer workflow. Library construction (I) ligates 454-specific adapters to DNA fragments (indicated as A and B) and couples amplification beads with DNA in an emulsion PCR to amplify fragments before sequencing (II). The beads are loaded into the picotiter plate (III).
(B) Schematic illustration of the pyrosequencing reaction which occurs on nucleotide incorporation to report sequencing-by-synthesis.

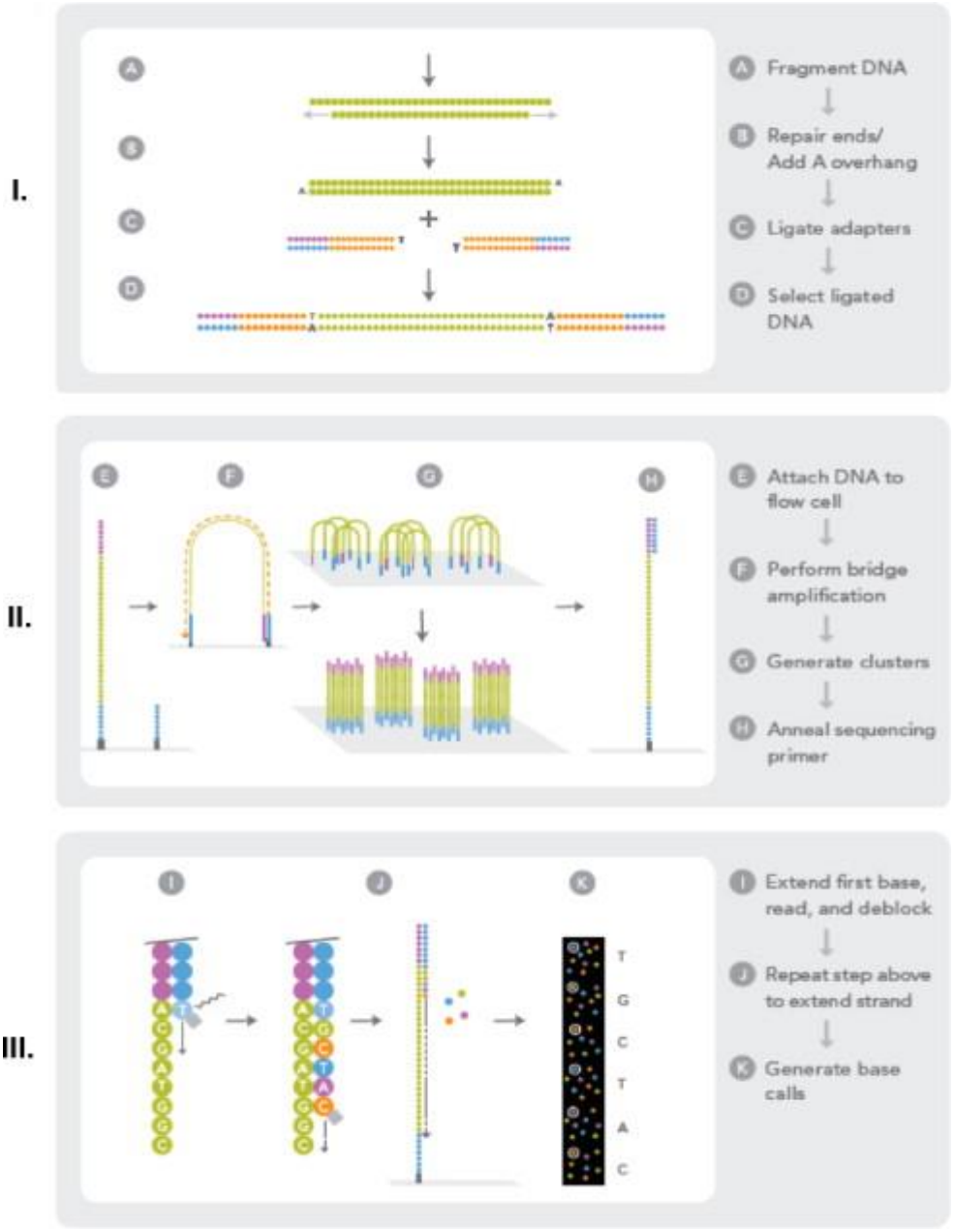
Adapted from http://www.454.com, Ansorge (2009)

Illumina (Solexa) sequencing

As described by Ansorge (2009), the principle of the Illumina sequencing method is also based on the sequencing-by-synthesis chemistry. Both ends of the DNA fragments are ligated to the adapters, and following denaturation, the DNA fragments are immobilised at one end on a solid support. The surface of the support is coated densely with primary and complementary adapters and each single-stranded fragment creates a 'bridge' structure by hybridising its free-end to the complementary adapter on the surface of the support. A mixture containing PCR amplification reagents is added, and adapters on the surface act as primers for the following PCR amplification. After several PCR cycles, random clusters of about 1000 copies of single-stranded DNA fragments are created on the surface (termed as polonies). The reaction mixture for the sequencing reactions and DNA synthesis is supplied onto the surface and contains primers, four reversible terminator nucleotides (each labelled with a different fluorescent dye) and the DNA polymerase. After incorporation into the DNA strand, the terminator nucleotide is detected and identified via its fluorescent dye. The terminator group at the 3′-end of the base and the fluorescent dye are then removed from the base and the synthesis cycle is repeated.

The sequence read length achieved in the repetitive reactions is about 35 nucleotides. However, sequences of at least 40 million polonies can be simultaneously determined in parallel, resulting in a very high sequence throughput, in the order of gigabases (Gb) per support. The system generates at least 1.5 Gb of single-read data per run, at least 3 Gb of data in a paired-end run, recording data from more than 50 million reads per flow cell (Ansorge, 2009). Moreover, the sequencing run time is decreased to 2-4 days, making it the most widely used next-generation sequencing platform to date (Schuster, 2007, Nelson *et al.*, 2014).

Figure 2.5. Schematic diagram of the Illumina Sequencing platform.



Legend: The outline of the Illumina Genome Analyser workflow shows: Similar fragmentation and adapter ligation steps take place, before applying the library onto the solid surface of a flow cell (I). Attached DNA fragments form 'bridge' molecules, which are subsequently amplified via an isothermal amplification process, leading to a cluster of identical fragments that are subsequently denatured for sequencing primer annealing (II). Amplified DNA fragments are subjected to sequencing-by-synthesis using 3′ blocked labelled nucleotides (III).

Adapted from http://www.solexa.com, Ansorge (2009)

2.3.4.2 Structure and diversity of the equine intestinal microbiome

In horses, fermentation of the ingested feed occurs due to the predominance of intestinal microbiota throughout the GI tract. High amounts of VFAs are produced in the large intestine (hindgut) when compared to stomach and small intestine of the horse (Figure 2.6), indicating that the majority of fermentation occurs in the hindgut (Al Jassim, 2006).

Figure 2.6. Products of fermentation shown as the concentration of VFA in different segments of the gastrointestinal tract of horses.

Legend: DRS, dry-rolled sorghum; SFS, steam-flaked sorghum; NG-S, non-glandular region of the stomach; G-S, glandular region of the stomach; SI-D, small intestine, duodenum; SI-JI, small intestine, jejunum and ileum; Cae, caecum; Col, colon; Rec, rectum.

Al Jassim (2006)

Comparative studies on the metabolites of fermentation in the compartments of the large intestine of horses and ponies have shown some differences in the quantities of total VFAs produced in the caecum, right dorsal colon and the faeces (equivalent to the rectum) (Dougal *et al.*, 2012). Significantly higher amounts of total VFAs, especially acetic acid, were observed in the right dorsal colon when compared to caecum and faeces (Table 2.5), indicating that higher proportions of microbiota inhabit the caecum and colon.

Table 2.5. Comparison of the VFAs and ammonia concentrations in the caecum, right dorsal colon and faeces of horses and ponies.

Dougal *et al*., (2012)

Intestinal microbiota are divided into three domains, Bacteria (*Eubacteria*), Archaea (*Archaeobacteria*) and eukaryotes. Bacteria and Archaea are classified as prokaryotes (single-celled organisms that lack a membrane bound nucleus, organelles and mitochondria), and other organisms such as protozoa (ciliates) and fungi are classified as eukaryotes (organism whose cells contain a nucleus and other organelles enclosed within membranes). Within the large intestine of the horse, significant differences in the microbial diversity (Table 2.6) and absolute quantity of bacteria, archaea and protozoa (Table 2.7) have been observed between the hindgut compartments and the faeces (Dougal *et al.*, 2012).

Table 2.6. Bacterial diversity in the large intestine, calculated using T-RFLP amplicons of 16S rRNA genes of microbial DNA extracted from the contents obtained from the caecum, right dorsal colon and faeces of horse and ponies.

Dougal *et al*. (2012)

Table 2.7. Absolute quantity of bacteria, and the quantity of archaea, protozoa and fungi relative to the total bacteria found in the caecum, right dorsal colon and faeces of horses, revealed by q-PCR.

Dougal *et al*., (2012)

Among the three domains, bacteria are the most abundant organisms found in the intestinal microbiome (Dougal *et al.*, 2012). While bacteria inhabit the entire GI tract, protozoa, fungi and viruses have been found only in the hindgut (Sadet-Bourgeteau and Julliand, 2010). Studies using metagenomics to investigate the microbial communities in the equine hindgut or faeces have reported large numbers of bacterial species in each sample, but the rarefaction curves of the observed species do not reach an asymptote, indicating that the richness of the bacterial community may be larger than identified in the studies (Costa *et al.*, 2012, Shepherd *et al.*, 2012). Advances in molecular and sequencing techniques have improved the knowledge of the structure and diversity of the microbial communities in the equine hindgut. However, the reporting of previously unidentified or unclassified species in recent studies suggests that novel information is still emerging, and a large proportion of microorganisms in the equine large intestine yet to be identified (Dougal *et al.*, 2013).

Intestinal Bacteria

According to the list of prokaryotic names with standing nomenclature (LPSN), the domain Bacteria is subdivided into 30 phyla and the domain Archaea has 5 phyla (Euzéby, 1997, Parte, 2014). Most intestinal bacteria are classified as cocci and rods, which stain either Gram positive or Gram negative. Examination of faecal samples from horses by microscopy has shown that the most common intestinal bacteria are Gram negative rods and cocci, the percentage counts of which were reported to be similar among native and light breeds of horses, but different when faecal samples were compared between seasons (Kobayashi *et al.*, 2006) (Table 2.8).

Table 2.8. Faecal bacteria and morphological enumeration in Hokkaido native horses and light horses using microscopy.

Legend: Each value represents the average of four horses. The values followed by different letters within the same column for each breed differ significantly ($P<0.05$).

Kobayashi *et al*., (2006)

Bacteria isolated from the hindgut of horses are predominantly anaerobes, which utilise multiple substrates available from the ingested feed. Some of the major groups of bacteria, each of which contain several genera and species, include cellulolytic (digest cellulose), hemicellulolytic (digest hemicellulose), amylolytic (digest starch), proteolytic (digest proteins), sugar utilising (utilise monosaccharides and disaccharides), acid utilising (utilise lactic, succinic, malic acids, etc.), ammonia producers, vitamin synthesisers, and methane producers (methanogens) (Hobson and Stewart, 1997). The fermentative properties of some bacteria isolated from the rumen (and also found in the hindgut of horses) are shown in Table 2.9.

Table 2.9. Fermentative properties of some bacteria isolated from the rumen.

Species	Function*	Products¶
Fibrobacter (Bacteroides) succinogenes	C,A	F,A,S
Ruminococcus albus	C,X	F,A,E,H,C
Ruminococcus flavefaciens	C,X	F,A,S,H
Butyrivibrio fibrisolvens	C,X,PR	F,A,L,B,E,H,C
Clostridium lochheadii	C,PR	F,A,B,E,H,C
Streptococcus bovis	A,S,SS,PR	L,A,F
Ruminobacter (Bacteroides) amylophilus	A,P,PR	F,A,S
Prevotella (Bacteroides) ruminocola	A,X,P,PR	F,A,P,S
Succinimonas amylolytica	A,D	A,S
Selenomonas ruminantium	A,SS,GU,LU,PR	A,L,P,H,C
Lachnospira multiparus	P,PR,A	F,A,E,L,H,C
Succinivibrio dextrinosolvens	P,D	F,A,L,S
Methanobrevibacter ruminantium	M,HU	M
Methanosarcina barkeri	M,HU	MC
Treponema bryantii	P,SS	F,A,L,S,E
Megasphaera elsdenii	SS,LU	A,P,B,V,CP,H,C
Lactobacillus sp.	SS	L
Anaerovibrio lipolytica	L,GU	A,P,S
Eubacterium ruminantium	SS	F,A,B,C
Oxalobacter formigenes	O	F,C
Wolinella succinogenes	HU	S,C

Legend: * C- cellulolytic; X- xylanolytic; A- amylolytic; D-dextrinolytic; P-pectinolytic; PR-proteolytic; L-lipolytic; M-methanogenic; GU-glycerol-utilising; LU-lactate-utilising; SS-major soluble sugar fermenter; HU- hydrogen-utiliser; O-oxalate-degrading.

¶ F- formate; A- acetate; E-ethanol; P-propionate; L- lactate; B-butyrate; S- succinate; V-valerate; CP- caproate; H- hydrogen; C- carbon dioxide; M-methane.

Russell and Hespell (1981)

Bacteria in the stomach and small intestine (foregut)

The equine stomach harbours an abundant and diverse mucosal microbiota that varies by individual (Perkins *et al.*, 2012). Previous culture-based studies have reported considerable amounts of bacteria (~10^6 - 10^8 CFU/ml) present in the stomach of the horse despite the acidic environment, which mainly include Lactobacilli, Streptococci and some other lactate-utilising bacteria (Varloud *et al.*, 2007a). Among the cultured microbes, Lactobacillus bacteria dominated the microbial community (de Fombelle *et al.*, 2003). Subsequent molecular-based studies have identified four major bacterial types, including *Lactobacillaceae, Streptococcaceae, Veillonellaceae* and *Pasteurellaceae* (St-Pierre *et al.*, 2013). Some species of symbiotic Lactobacilli isolated from the stomach of the horse include *Lactobacillus salivarius, L. crispatus, L. reuteri* and *L. agilis* (isolated by culture from the non-secreting area

of the stomach) (Yuki *et al.*, 2000) and *L. mucosae* and *L. delbrueckii* (identified by molecular techniques from the stomach of roughage-fed horses) (Al Jassim *et al.*, 2005).

Strictly anaerobic bacteria colonise the small intestine of the horse, with numbers ranging from 10^6 - 10^9 CFU/ml, and are mainly represented by *Lactobacilli*, *Enterobacteria*, *Enterococci*, *Streptococci* and other lactate-utilising bacterial species. Whereas *Lactobacilli* are dominant in the culture of stomach contents, *Streptococci* seem to dominate the culture of small intestinal contents (de Fombelle *et al.*, 2003). Recent next generation sequencing studies have shown that the microbiota of the ileum (small intestine) is significantly less diverse and the core community is smaller than that of the large intestine (caecum and colon) (Dougal *et al.*, 2013), perhaps due to the rapid transit of digesta through the small intestine compared to the large intestine. Similarly, the species richness (number of operational taxonomic units (OTUs)) and bacterial load (determined by qPCR) is lowest in the ileum. Firmicutes (70%) were identified as the most dominant ileal bacterial phylum, followed by Proteobacteria (14%) and Bacteroidetes (10%) (Dougal *et al.*, 2013).

Bacteria and archaea in the large intestine (hindgut)

The large intestine has an environment that is highly favourable for colonisation by microbiota, due to the a neutral pH and the slow passage rate of digesta (longer retention time) (Julliand, 2005). The microbiota are compartment specific with a clear separation at the pelvic flexure of the colon (Moore and Dehority 1993). Examination of caecal, colonic and faecal contents have shown that the microbial density, diversity and species richness tends to differ between more proximal regions of the hindgut and the faeces (Daly *et al.*, 2001, Julliand *et al.*, 2001, Dougal *et al.*, 2012, Dougal *et al.*, 2013). The differences in microbial diversity may suggest that the microbial activity and function of the caecal ecosystem differs from that of the colon and faeces, but interpretation of these data is difficult due to variations in the measuring techniques and diets fed to the horses. More recent studies using high-throughput sequencing techniques have shown a significant increase in diversity towards the distal gut, with a stable bacterial population observed from the cecum through to the faeces. The results of these studies suggest that faecal samples show strong stability of microbiota populations and may represent changes occurring in the distal compartments of the equine GI tract (Blackmore *et al.*, 2013, Costa *et al.*, 2015a).

Cellulose degrading bacteria play a major role in the digestive function of the horse. Culture-based studies have shown that the numbers of cellulolytic bacteria vary from 10^4-10^7

CFU/ml in caecal and colonic contents (Kern *et al.*, 1973, Julliand *et al.*, 2001, de Fombelle *et al.*, 2003). *Streptococci* and *Lactobacilli* are considered the main glycolytic and amylolytic bacteria within the hindgut, with concentrations varying from 10^3-10^6 CFU/ml in cultures of caecal contents (Bailey *et al.*, 2003b, Al Jassim *et al.*, 2005). Proteolytic bacteria have been identified in the caecum and colon (10^8-10^9 CFU/ml (Mackie and Wilkins, 1988). Daly *et al.*, (2001) described *Clostridium* spp., *Ruminococcus* spp., *Butyrivibrio* spp. and *Eubacterium* spp. as the most important cellulolytic bacteria using molecular-based techniques. Caecal samples had significantly lower *R. flavefaciens* and *F. succinogenes* concentrations than samples from the ventral and dorsal colon, and *Streptococcus bovis* was more numerous in the luminal contents of the ventral and dorsal colon than in the caecum, indicating that both the caecum and colon play important roles in fibre degradation (Hastie *et al.*, 2008). *Streptococcus bovis* and *S. equinus, Lactobacillus salivarius, L. mucosae, L. delbrueckii and Mitsuokella jalaludinii* were reported to be dominant lactic acid producing bacteria, while *Veillonella* spp. and *Megashpera* spp. are the main lactate-utilising inhabitants of the hindgut (Al Jassim *et al.*, 2005).

Over the past decade, studies have attempted to characterise the equine hindgut bacterial community using culture-independent methods, some of which used invasive techniques such as cannulation or were terminal and collected representative samples from the hindgut after euthanasia (Daly *et al.*, 2001, Daly and Shirazi-Beechey, 2003, Hastie *et al.*, 2008), whereas others were non-invasive and examined faecal samples (Yamano *et al.*, 2008, Earing *et al.*, 2012, Shepherd *et al.*, 2012, Steelman *et al.*, 2012). Most studies have focused on the bacterial population, and have given minimal attention to the archaea, protozoa and fungi microbial communities.

Shepherd and co-workers (2012) were the first group to utilise 454 pyrosequencing and metagenomics involving high-throughput next-generation sequencing, to study the faecal bacterial community of forage-fed horses. The bacterial sequences generated in the study were assigned to 16 phyla, 10 of which were present in all samples (two samples pooled from two horses). The most dominant phylum reported was Firmicutes (70%), followed by Verrucomicrobia, Proteobacteria and Bacteroidetes. Several less abundant phyla were also identified in the study, such as Actinobacteria, Cyanobacteria and TM7. Another study (Costa *et al.*, 2012) compared the faecal microbiome of healthy horses and horses with idiopathic colitis, and reported that Firmicutes (68%) were the most abundant bacterial phyla identified in the faeces of healthy horses, followed by Bacteroidetes, Proteobacteria and other less

abundant bacterial phyla. In both the studies, sequence data on a spectrum of microbiota found in the equine hindgut, were obtained by pyrosequencing of 16S rRNA gene amplicons within the V4 region (Shepherd *et al.*, 2012) and V3-V5 regions (Costa *et al.*, 2012), and the results from both studies agreed that members of the Firmicutes phylum dominated the faecal microbiota (Table 2.10).

Table 2.10. The distribution of equine faecal microbiome at a phylum level as assessed by next generation sequencing of 16S rRNA gene PCR products, % calculated after excluding unclassified sequences.

	Reference	
Method	Shepherd *et al*., 2012 V4 region	Costa *et al*., 2012 V3-V5 region
Firmicutes	70%	64%
Bacteriodetes	6%	14%
Proteobacteria	6%	12%
Actinobacteria	3%	4%
Spirochaetes	NR	3%
Verrucomicrobia	7%	NR
TM7	3%	NR

*NR - not reported

Costa and Weese (2012)

Many other subsequent studies on the hindgut or faceal microbiota in horses have reported similar findings (Dougal *et al.*, 2012, Steelman *et al.*, 2012, Blackmore *et al.*, 2013, Dougal *et al.*, 2013, Dougal *et al.*, 2014). Some of the differences in phyla distribution observed between the studies may be explained by the differences in the types of horses and their feeding management during the study period.

The results of phylogenetic analysis indicated that Clostridiales members of the Lachnospiraceae family, were the most abundant bacteria shared between horses, suggesting that this group of microbes forms an important part of the core bacterial population of healthy horses (Costa *et al.*, 2012). The core bacterial community (bacteria present at ≥0.1% relative abundance) within the large intestine of the horse was explored further using 454 pyrosequencing, and a core group of bacterial families was identified in the hindgut regions (Dougal *et al.*, 2013). The most abundant member in the ileum was the Lactobacillaceae, in the proximal hindgut the Lachnospiraceae, and in the distal hindgut the Prevotellaceae. This

presence of low abundances of microbial communities was thought to be associated, in part, with the susceptibility of some horses to digestive upsets.

The core bacterial community in the large intestine was much smaller (5-15% of the total number of sequences), and showed a different pattern to that of the ileum (Figure 2.7). A larger number of OTUs were present in low abundance (the largest single OTU in any region of the large intestine accounts for only 2% of all sequences for that region), compared to the ileum, which was made up of approximately 32% of all sequences comprising of only seven OTUs of varying abundance (Dougal *et al.*, 2013) (Figure 2.7). Clostridiales are core components of the equine intestinal microbiota (Costa and Weese, 2012). Only *C. difficile* and *C. perfringens* are known to be enteropathogenic in animals, whereas most members of this order are important cellulolytic bacteria with diverse functions in the digestive system (Songer, 1996). A decrease in the abundance of Clostridiales was also seen in horses affected with acute colitis, indicating a potential link of this order to GI disease (Costa *et al.*, 2012).

Figure 2.7. The core bacterial community in different compartments of the horse's large intestine.

Legend: The core community is defined as those OTUs (Operational taxonomic units, clustered at 97% similarity) present in all ten animals and which abundances are 0.1% (or greater) of the total number of sequences in that gut region. The lower pale blue section of the bar indicates the proportion that is not part of the core. The remaining individual coloured sections represent each OTU of which the core is comprised; Ileum (7), caecum (31), right ventral colon (RVC) (33), left ventral colon (LVC) (28), left dorsal colon (LDC) (12), right dorsal colon (RDC) (18), small colon (16) and faeces (25).

Dougal *et al*., (2013)

Inter-horse variation (differences between individual horses) in the relative abundance of bacterial communities identified in the hindgut or faeces has been reported in several studies (Costa *et al.*, 2012, Dougal *et al.*, 2012, Steelman *et al.*, 2012, Schoster *et al.*, 2013). In the first study (Costa *et al.*, 2012), the proportion of Firmicutes was reported to range from 39-78% in the group of healthy horses, and was lower in two horses (mean 44%) as compared to the other four horses (mean 74%). The former two horses were housed together at the University's Teaching Veterinary Hospital and were fed grass-hay only, while the latter four were maintained on mixed diets containing pelleted feed and forage, and each horse was

located on a different farm. The proportion of Proteobacteria (37- 43%) was also higher in the two University teaching horses, as compared to 3% in the other horse.

In the second study (Steelman *et al.*, 2012) that characterised the bacterial communities in healthy horses and those with chronic laminitis, the bacterial community was dominated by Firmicutes (69% in healthy horses and 57% in the laminitis group of horses), followed by other phyla, but there was a high level of individual horse variation at all taxonomic ranks. In this study, faecal samples from horses with chronic laminitis had a higher bacterial diversity than observed in the faeces obtained from healthy horses. However, the horses in the healthy group (n=10) were of one breed (Quarter Horses) with a mean age of 8 years, whereas horses in the laminitis group were of various breeds of horses (n=7) and one pony, that were >15 years of age. The two groups of horses were maintained on separate farms providing different dietary management (alfalfa for the healthy horses and hay for the laminitis group of horses), and these differences in animal selection and management perhaps contributed to the variation observed in the bacterial communities.

A third study conducted by Dougal and co-workers (2012) utilised a mixed population of Thoroughbred horses (n=3) and British native-bred ponies (n=6) that were maintained on different diets, some fed supplementary feeds while others were on grass-only diets, which may have confounded the results. Nonetheless, large variations were also seen when microbial populations were compared between six horses with similar dietary and housing management (Schoster *et al.*, 2013). The observed variation in findings indicated that dietary and management factors have a significant impact on the intestinal microbiome of healthy horses.

Most studies that have used next-generation sequencing techniques to characterise the hindgut or faecal microbiota in horses have also reported high diversity in the microbiota which was dominated by the phylum Firmicutes, and Lachnospiraceae and Ruminococcaceae were the most abundant families of cellulolytic bacteria classified under the order Clostridiales (Shepherd *et al.*, 2012, O' Donnell *et al.*, 2013). However, some bacterial sequences detected in these studies remained unidentified or not yet classified, perhaps because the sequences have been under-represented in the universal public domains/ databases (Daly *et al.*, 2001). This lack of recognised sequences reflects the diversity in the equine microbiota and suggests that the profile could contain some novel bacterial species, which needs further investigation.

Whilst most studies have investigated the hindgut microbiota of horses by using samples collected from faecal material (Kobayashi *et al.*, 2006, Yamano *et al.*, 2008, Shepherd *et al.*, 2012), it is still unclear as to how representative faecal microbiota are (in terms of diversity and relative abundance of microbial species) to more proximal regions in the equine hindgut (caecum or colon), as discussed in section 2.3.4.1. Certain bacteria (*Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Ruminococcus albus*) identified in the faeces have also been identified in the hindgut (Hastie *et al.*, 2008). But, 16S rRNA gene dendrograms and qPCR, the bacterial diversity of the caecum was shown to cluster separately from other gut regions (Dougal *et al.*, 2012). The relative abundance of four bacterial families identified as part of the core microbiome were also shown to be represented in faecal and hindgut samples, but many other bacteria identified in the caecum and colon were not found in the faecal samples (Figure 2.8) (Dougal *et al.*, 2013), supporting the argument that the microbial diversity may differ between the hindgut compartments.

Nevertheless, studies using molecular methods to explore the microbiome have shown contradictory results, with one study reporting that faecal samples were more likely to represent the microbial population of the right dorsal colon rather than the caecum (Dougal *et al.*, 2012). In contrast another study reported the highest similarity when faecal samples were compared to caecal samples (Schoster *et al.*, 2013). Both the investigators concluded that careful consideration is warranted when planning microbial investigations of the equine hindgut using faecal samples. All of the aforementioned studies suggested that further investigation into the factors that influence an individual horse's gut microbiome are required, and that general management factors and the site for sample collection must be considered when designing, interpreting and comparing data obtained from microbiome studies.

Figure 2.8. Relative abundance of the most prevalent bacterial families identified as the core bacterial community in horses.

Legend: Regions of the large intestine included are the caecum, right ventral colon (RVC), left ventral colon (LVC), left dorsal colon (LDC), right dorsal colon (RDC), small colon and faeces. Values presented do not account for all members of stated families found in each gut region, only those identified as being part of the core; error bars represent the standard deviation.

Dougal *et al.,* (2013)

Ciliate protozoa and cellulolytic fungi

Gut protozoa are anaerobic, live within a large bacterial population, and have the ability to ferment the structural polysaccharides of plants. Ciliated protozoa predominantly inhabit the caecum and colon because the digesta retention time exceeds the protozoan generation time and the pH is neutral (Dehority, 1986). Protozoa are specific to the host, intestinal compartment and diet, which is evident by the higher density and species richness of ciliate protozoa found in horses compared to donkeys and mules (Gürelli, 2012), the higher diversity of protozoan species observed in the large colon than in the caecum of the horse (Dehority, 1986), and higher numbers observed in ponies fed hay rather than fed concentrate feed (Goodson *et al.*, 1988). Over 50 protozoan species have been described in the hindgut and faeces of horses, but similar to the bacterial community, large individual variations have been observed in the abundance of protozoa between horses (Hsiung, 1930, Kern *et al.*, 1974, Goodson *et al.*, 1988, Moore and Dehority, 1993). The distribution and counts of ciliate protozoa fluctuate from 10^2 - 10^5 units/ml, depending on the different regions of the hindgut, with the highest concentrations reported in the right dorsal colon (Moore & Dehority 1993). Protozoa are more abundant than archaea and fungi (Table 2.7), but studies on the diversity and community structure of ciliate protozoa in the hindgut of horses, examined using molecular techniques are limited (Dougal *et al.*, 2012).

Other microbiota, such as fungal zoospores, have been identified in the caecum, and these fungi are useful in the degradation of plant fibres by the fermentation of polysaccharides in plant cell walls (Trinci *et al.*, 1994). The majority of fungal strains isolated from the caecum are anaerobic cellulolytic phycomycete fungi, belonging to the genus *Piromyces*, with four species identified (Orpin, 1981). The *Piromyces* strains found in the equine caecum (10 - 10^4 CFU/ml) degraded cellulose more rapidly and to a greater extent, compared to the strains found in ruminants (Julliand *et al.*, 1998). The equine strains were observed to grow more rapidly on glucose and cellobiose and did not produce lactate (Orpin, 1981, Julliand *et al.*, 1998). Studies on the diversity and abundance of fungi in the hindgut of horses using molecular techniques are limited (Liggenstoffer *et al.*, 2010, Dougal *et al.*, 2012).

2.3.4.3 Alterations in gut microbiota

Age and breed

During the early developmental stages of neonatal horses, the establishment of an intestinal bacterial community was observed to be a sequential process, with complex microbiota established by the first day of life, and microbiota adaptations occurring during the first month (Earing *et al.*, 2012, Faubladier *et al.*, 2014, Costa *et al.*, 2015b). The microbiota of foals older than 60 days resemble that of the mare, although differences in community membership are still present (Costa *et al.*, 2015b). However, it remains to be determined whether this initial colonisation is affected by the diet and management of the mare and foal

The age of the horse affects the population of microbiota in the hindgut, with a reduction in diversity reported in elderly horses compared to mature adult horses (Dougal *et al.*, 2014). The breed or type of horse may also influence the population of microbiota in the hindgut (Kobayashi *et al.*, 2006, Yamano *et al.*, 2008). This theory was supported by the finding of a higher relative weight of the caecum and colon in native breeds of horses when compared to the light breeds (Kobayashi *et al.*, 2006). Another study in a similar group of horses showed that Hokkaido native horses had a higher microbial diversity than light breeds of horses (Yamano *et al.*, 2008). The anatomical differences in the hindgut and the variation in microbial diversity were suggested to be the reasons why native horses adapted better to the lower plane of nutrition than light horses.

Diet and Management

Diet appears to have a greater influence on the structure of bacterial communities in the hindgut, than the age or breed of the horse, and the management of horses at different stages of growth may affect the population of the hindgut and faecal microbiota. Early studies that investigated the effects of diet on the hindgut and faecal microbiota indicated that the microbiota profiles were dissimilar in horses fed different diets or when managed differently. For example, temporal variations in the faecal bacterial and protozoal counts were observed in Hokkaido native and light breeds of horses that were maintained on woodland pasture in winter and grassland pasture for the rest of the year, using identification via microscopy and Gram's staining (Kobayashi *et al.*, 2006). The study also reported that the total VFA pattern shifted to more acetate and less propionate production in winter.

Culture-based studies have shown that abrupt dietary change from hay to concentrate feeding resulted in increased numbers of total viable anaerobic bacteria and protozoa, which resembled those that occur in the rumen under similar feeding conditions (Goodson *et al.* 1988). The concentration of total anaerobes and lactic acid utilising bacteria increased, and cellulolytic bacteria decreased in the cecum of horses fed the high-starch diet (Medina *et al.*, 2002). Variable amounts of barley (30-50%) incorporated in the hay diet resulted in an increase in amylolytic bacteria such as *Lactobacilli* and *Streptococci* in the caecum and the colon, which was observed ~29 h after the change in diet, and persisted over 14 days of feeding the diet (de Fombelle *et al.*, 2001, Julliand *et al.*, 2001). In forage-fed horses, the faecal quantities of *Staphylococcus* and *Lactobacillus* species that increased linearly when horses were fed incremental quantities of grain in the diet, decreased when the horses were returned to feeding exclusively on pasture (van den Berg *et al.*, 2013). These shifts in microbiota populations were also associated with increased lactic acid concentrations, decreased hindgut pH and acetate + butyrate: propionate ratio, and consequent microbiota disturbances (de Fombelle *et al.*, 2001, Willing *et al.*, 2009).

Willing and co-workers (2009) used a molecular technique (T-RFLP) to compare the microbiota in caecal contents obtained from horses fed a high-energy forage diet (comprising of early cut timothy/meadow fescue haylage), to horses that were fed a mixed diet (comprising of late cut timothy/meadow fescue and concentrates mix of oats, soy bean meal, wheat bran and sugar). They reported a dynamic shift in the proportion of Firmicutes and Bacteroidetes identified in the caecal contents. The microbiota of forage-fed horses comprised nearly 50% Bacteriodetes and 46% Firmicutes, whereas in horses supplemented with grain, the proportion of Bacteroidetes decreased to 27% and the Firmicutes increased to 73% of the bacterial sequences identified (Willing *et al.*, 2009). Although there appeared to be no effect of the forage diet on the bacterial diversity in the hindgut and faeces, Willing *et al.* (2009) showed that the absence of concentrate from the diet resulted in a more stable bacterial population, even though the level of dietary starch utilised in the study did not initiate clinical signs of digestive disturbance in the horses. The presence of *Lactobacillus ruminis* was observed only in horses fed the concentrate-mixed diets and significantly lower counts of lactic acid bacilli, especially the *Streptococcus bovis*/ *equinus* complex, were observed in the caecal contents of horses fed the forage only diet.

The bypass of starch into the hindgut and consequent microbiota disturbance has been implicated in the increased risk of colic and laminitis in horses (Durham, 2009, Frank *et al.*,

2010). However, horses that were fed conserved forage diets such as silage, haylage and hay (harvested and manufactured from the same grass crop to have variable moisture content of the order silage > haylage > hay), tended to have limited microbial changes in the colon and faeces (Müller *et al.*, 2008, Muhonen *et al.*, 2009). Therefore, it has been suggested that the consistently low levels of Streptococcal spp. in the faeces of horses fed forage could perhaps support the use of a forage diet in horses that are susceptible to GI disturbances (Willing *et al.*, 2009).

More recently, Dougal and co-workers compared the core microbiomes of horses fed three diets, namely hay (representing a high fibre diet), oil (representing a high oil/ fat diet) and CHO (carbohydrate, representing a high starch diet). They reported that the core microbiome was dominated by members of the Firmicutes phylum, and Lachnospiraceae was the most abundant bacterial family identified in the core, irrespective of the diet fed to the horses. Interestingly, the size of the core microbiome associated with the forage only diet was 16%, whereas when oil was supplemented in the diet, the core reduced in size to 10% and was further reduced to 5% when starch was supplemented in the diet (Dougal *et al.*, 2014). They also reported significant differences in the OTUs identified between horses fed the different diets (Figure 2.9).

Figure 2.9. Phylogenetic tree showing significant OTUs for diet.

Legend: The radial phylogenetic tree shows only those OTUs (operational taxonomic units) found to be significant (corrected P<0.1) and was built using the function UPGMA (Unweighted pair group method with arithmetic mean). The coloured outer ring indicates the bacterial phyla of the OTU classification, while the outer circles show the relative abundances of each OTU for the different diets; Hay- high fibre diet (green circles), Oil - high oil diet (pink circles) and CHO (carbohydrate) - high starch diet (blue circles).

Dougal *et al*., (2014)

An *in vitro* model of starch induction involving sequencing of the 16S rRNA gene obtained from faecal bacterial communities, showed that members of the Veillonellaceae family were the most abundant lactate-utilising bacteria, which increased from an abundance of ≤1% before the starch induction (Biddle *et al.*, 2013). *Megasphaera elsdenii* (strains of which have been shown to be effective in preventing lactic acidosis in cattle and have been developed as a probiotic therapy (Aikman *et al.*, 2011)), was highly abundant in all starch and starch-lactate cultures in which lactate was attenuated. However, it was unclear as to why these bacteria did not proliferate in all horses, and it was suggested that other factors such as pH and competitive microbial interactions exert selective pressure under starch or lactate conditions (Biddle *et al.*, 2013).

Many studies investigating the effects of diet or disease on the hindgut or faecal microbiota in horses have reported individual variation between the microbiota profiles of the horses used in the studies (Costa *et al.*, 2012, O' Donnell *et al.*, 2013, Dougal *et al.*, 2014). Variations occur within an individual due to environmental factors, such as diet or management changes, however there appears to be a strong stability in the microbiota profiles within an individual animal (Blackmore *et al.*, 2013). The amount of variation in the microbiota profiles between horses fed the same diet has not been quantified and this requires further investigation.

Transport, fasting, anaesthesia, antibiotics and microbial supplements

Transportation and fasting are routine management practices for competition horses, which can affect the gut microbiota. Two studies using molecular and next-generation sequencing techniques have demonstrated that transportation of healthy horses resulted in disturbance of the faecal bacterial ecosystem (Faubladier *et al.*, 2013, Schoster *et al.*, 2015). Regardless of dietary treatment, the faecal microbiota profiles were significantly different before and after transportation (Faubladier *et al.*, 2013). There were significant changes in the relative abundances of bacterial phyla, classes, orders, and families after transport, fasting, and anaesthesia. Most notably horses had a significantly lower abundance of Clostridiales after transport compared with baseline, a decreased abundance of Rickettsiales after fasting, and anaesthesia also had a significant effect on bacterial community membership and structure (Schoster *et al.*, 2015). These findings indicate that these common management practices are potentially stress factors for the equine intestinal microbiome. However, the effect of other management practices, such as restricted versus free grazing, withholding feed for prolonged

periods, and confinement of horses in stables or yards, has not been examined, and requires investigation.

Systemic antimicrobial drugs such as procaine penicillin, ceftiofur sodium and trimethoprim sulfadiazine, administered via parenteral or oral route, affect the intestinal microbiota of horses (Costa *et al.*, 2015c). A significant decrease in microbiota richness and diversity was observed after treatment, with significant changes in the population structure and community membership of faecal bacteria of horses. Changes in structure and membership caused by antimicrobial administration were specific for each drug, with the largest effect seen in trimethoprim sulfadiazine due to a drastic decrease in the relative abundance of the phylum Verrucomicrobia in the faeces. Twenty-five days after the end of treatment, faecal bacterial profiles were returning to pre-treatment patterns indicating a recovery from changes caused by antimicrobial administration. However, differences in the bacterial community membership were still evident, indicating that the effects of antimicrobials on the faecal microbiota of horses may be prolonged (Costa *et al.*, 2015c). Several other drugs (such as anti-inflammatories, anaesthetics, spasmolytics, and cathartics) are commonly administered along with antimicrobials during medical treatment and surgical procedures, but their effects on the gut microbiota and the variation between individual horses during the convalescent period is unknown, and this requires research attention (Grønvold *et al.*, 2010).

A practical approach utilising microbes to alleviate GI disturbances in performance horses has been suggested with the administration of probiotics and prebiotics. Supplementing the diet with probiotics could help reduce the negative impact of diet and management practices (such as fasting and transportation) on the faecal bacterial ecosystem. For example, when the diet of horses was supplemented with fructo-oligosaccharide (a prebiotic), alterations were observed in the microbiota populations, faecal pH and VFA concentrations (Berg *et al.*, 2005). Addition of a probiotic *Saccharomyces cerevisiae* preparation increased the concentration of viable yeast cells in the caecal and colonic contents, and although it had minimal effect on the bacterial counts in the caecum and colon, it modified the pH, lactic acid and VFA concentrations, which appeared to limit the extent of undesirable changes in the intestinal ecosystem of the horse (Medina *et al.*, 2002). Similarly, when the diet of horses subjected to transportation for 2 hours was supplemented with *Saccharomyces cerevisiae* (a probiotic), a greater similarity was observed between the pre- and post-transportation profiles of bacterial communities. The faecal concentrations of lactate-utilising bacteria and cellulolytic bacteria were greater in supplemented horses than in control horses, indicating that

supplementing the diet with beneficial microbes reduced the extent of disruptions occurring in the intestinal ecosystem (Faubladier *et al.*, 2013).

Disease

Some horses are more susceptible to GI disorders, and the reason for this predisposition may be linked to their gut microbiota (Costa *et al.*, 2012). Alterations in the hindgut microbiome appear to be associated with GI disease states due to the significant shifts in the population of gastrointestinal microbiota observed in clinical conditions such as colitis and carbohydrate-induced laminitis (Milinovich *et al.*, 2006, Costa *et al.*, 2012, Shepherd *et al.*, 2012, Steelman *et al.*, 2012).

Although there is individual horse-variation in the abundance of bacterial phyla identified in the faeces, the faecal microbiota of most healthy horses was dominated by the phylum Firmicutes (68%), followed by Bacteroidetes (14%), Proteobacteria (10%) and other less abundant phyla (Costa *et al.*, 2012). In contrast, Bacteroidetes (40%) was the most abundant phylum identified in the faeces of horses diagnosed with colitis. The abundance of Fimicutes (30%) reduced to half of the proportion identified in healthy horses, while the proportion of Bacteroidetes (40%) and Proteobacteria (18%) had nearly doubled (Figure 2.10). Based on these findings, it was hypothesised that colitis may be a disease of gut dysbiosis, rather than one that occurs simply through the overgrowth of an individual pathogenic microorganism (Costa *et al.*, 2012).

Figure 2.10. Faceal bacterial population in healthy horses compared to horses with colitis at a phylum level.

Costa *et al*., (2012)

Horses that were diagnosed with simple colonic obstruction and distension (a common dietary induced intestinal disease), showed progressive and significant increases in relative abundances of the Lachnospiraceae family, the Bacteroidetes phylum and members of the lactic acid producing bacillus-lactobacillus-streptococcus group, with a simultaneous decrease in the proportion of obligate fibrolytic, acid intolerant bacteria such as Fibrobacter and members of the Ruminococcaceae family (Daly *et al.*, 2012). Investigation of the faecal microbiota has shown that when the abundance of a particular microbial species increases, metabolic disease can be triggered (Julliand *et al.*, 2001, Bailey *et al.*, 2003a). For example, a significantly higher number of bacterial taxa were identified per individual, in the group of horses with laminitis (419 OTUs) as compared to healthy horses (355 OTUs) (Steelman *et al.*, 2012). An *in vivo* model of oligofructose-induced laminitis also showed an increase in the population of Streptococcal species 8 to 16 hours post oligofructose administration, followed by a rapid decline in pH and subsequent lameness at 24-32 hours (Milinovich *et al.*, 2006). The hindgut microbiota present before the onset of laminitis was predominated by the presence of oligofructose-utilising bacteria, and shifts in the population of *Lactobacillus* and *Escherichia coli* species were observed during the induced laminitis phase. This secondary shift in microbial populations was believed to have caused the death *en masse* and rapid cell lysis of the equine hindgut streptococcal species, resulting in liberation of cellular contents that perhaps triggered the onset of laminitis in the horses under study (Milinovich *et al.*, 2010).

The community structure of the faecal bacterial in horses diagnosed with equine metabolic syndrome (a condition associated with insulin resistance and laminitis) and colitis (an inflammatory condition of the colon) was shown to be significantly different to healthy horses (Figure 2.10 and 2.11) (Costa *et al.*, 2012, Elzinga *et al.*, 2016). Hence, it was hypothesised that, in animals that already have a chronically perturbed microbiota, susceptibility to disease conditions (such as acidosis, laminitis or colitis) may be higher (Longland and Byrd, 2006).

Figure 2.11. Bacterial population structure in equine metabolic syndrome (EMS) versus non-EMS control horses.

Legend: Representation of bacterial population structure as measured by Yue and Clayton similarity coefficient in n=10 EMS and n=10 non-EMS control horses.

Elzinga *et al*., (2016)

Other recent studies using high-throughput sequencing have demonstrated an association between the gut microbiota and several nutrition-related diseases in horses, colic and large colon volvulus (Venable, 2011, Venable *et al.*, 2013, Weese *et al.*, 2015), carbohydrate induced laminitis (Moreau *et al.*, 2014), chronic laminitis (Steelman *et al.*, 2012), equine grass sickness (Leng *et al.*, 2015), and gastric ulcers (Dong *et al.*, 2016). Associations between the gut microbiota and behavioural problems in horses have also been identified (Bulmer *et al.*, 2015, Destrez *et al.*, 2015).

2.4 Summary

The horse evolved as a free-grazing herbivore with a gastrointestinal tract designed to digest small quantities of soluble carbohydrates and protein, and maximise the digestion of fibre in the hindgut via microbial fermentation. Modern feeding and management practices such as confinement, feeding of concentrate meals, restriction of feeding times, and abrupt changes in dietary management, have subjected the domestic horse to digestive challenges that may differ from that of feral horses.

An increasing proportion of horses and ponies in modern societies are maintained for leisure purposes, and many of these animals are mixed breeds of native ponies that are efficient feed converters. Concerns regarding the levels of obesity in the leisure horse population have been highlighted in the current literature. However, information on the demographics of the leisure horse population in New Zealand in lacking, and the prevalence of obesity and other nutrition-related diseases in the leisure horse population in New Zealand is currently unknown.

Dietary changes are a common problem in intensively managed horses that are confined in stables, with limited access to grazing on pasture. There are strong indications of an association between dietary management and the gut microbiota in horses. However, the composition of the normal microbiota in healthy horses kept on pasture, and the effects of different types of forage diets on the gut microbial population in the horse are not well understood. The functional implications of changes in equine hindgut microbiota due to changes in dietary management are also unclear and warrant further investigation. Therefore, it is important to quantify the population of gut microbiota in horses fed different types of diets, and examine the dynamics of the microbiota population following dietary change.

2.5 Research Questions

Based on the literature reviewed, there are several aspects relating to the dietary management and faecal microbiota of horses that require further investigation. This thesis aims to address the following research questions:

1) Feeding management and prevalence of obesity in leisure horses in New Zealand:
    a. What are the typical feeding and management practices of Pony Club horses in New Zealand?
    b. What is the owner-reported prevalence of disease in this population?
    c. Is obesity prevalent in Pony Club horses in New Zealand?
    d. Do Pony Club horse-owners underestimate body weight and condition (obesity) in their animals?
    e. Do horses and ponies kept on pasture maintain body weight and condition between seasons?

2) Effects of diet and management on the gut microbiota of horses:
    a. What is the composition of the gut microbiota of horses grazing on pasture in New Zealand?
    b. Is there variation in the gut microbiota between horses?
    c. Does the type of diet and seasonal variation in dietary composition influence the population of gut microbiota in the horse?
    d. How quickly does the gut microbiota adapt to abrupt dietary changes in horses?
    e. Does the gut microbiota stabilise when diet and management are consistent?

2.5 Aims and hypotheses

This thesis aims to provide an original contribution to the current knowledge on the relationship between dietary management and the faecal microbiota of horses. The new knowledge may allow the development of feeding strategies to influence, or even modify, the faecal microbiota to reduce the occurrence of gastrointestinal disturbances, and potentially improve the health, performance, and welfare of horses.

The specific aims and hypotheses addressed in each chapter of this thesis are:

Chapter 3: To describe the demographic characteristics of Pony Club riders and their horses, the general feeding and management, and the owner-reported prevalence of nutrition-related health conditions in Pony Club horses in New Zealand.

Chapter 4: To describe and compare the body condition and morphometric measures of adiposity in a cohort of Pony Club horses in the lower North Island of New Zealand at two time points in the year.

Chapter 5: To describe and compare the faecal microbiota of yearling horses grazing on pasture with those fed exclusively on an ensiled chopped forage-grain diet in loose boxes, and to investigate the changes in the relative abundance of faecal microbiota following an abrupt dietary change from an ensiled chopped forage grain diet to pasture. The specific hypotheses tested were:

Hypothesis: Distinct and rapid changes will occur in the faecal microbiota of horses switched from one forage-based diet to another.

Hypothesis: The variation in faecal microbiota between diets will be greater than the variation between horses.

Chapter 6: To describe the faecal microbiota of mature adult horses grazing on pasture and supplemented with hay during the winter months, and to identify changes in the microbiota populations across seasons, and to correlate these changes with pasture composition and climatic variables. The specific hypotheses tested were:

Hypothesis: There will be significant differences in the diversity and relative abundance of faecal microbiota of horses on each diet.

Hypothesis: There will be seasonal differences in the faecal microbiota of normal horses maintained at pasture.

Chapter 7: To investigate changes in the transit time of digesta in horses fed four different forage-based diets, and to determine the effects of individual variation between horses on the transit time of digesta by using indigestible markers. The specific hypotheses tested were:

Hypothesis: The transit time of digesta in horses fed pasture will be shorter than in horses fed conserved forage diets.

Hypothesis: There will be significant differences in the feed intake and transit time of digesta between Thoroughbred horses managed under similar conditions on the same diet.

Chapter 8: To investigate changes in the faecal microbiota of horses following abrupt dietary transition, and to determine how quickly the bacterial communities respond to dietary change, and then stabilise when fed the same diet. The specific hypotheses tested were:

Hypothesis: The faecal microbiota of horses will be diet-specific.

Hypothesis: The response of faecal microbiota to dietary change will be associated with the transit time of the digesta.

Hypothesis: Diets that have a consistent nutrient composition will be associated with a more stable faecal microbiota.

CHAPTER 3

HEALTH AND MANAGEMENT OF PONY CLUB HORSES

(Online Cross-sectional Survey)

PRELUDE TO CHAPTER 3

Dietary and management practices are known to influence health, but there is limited published information on the feeding, health and management of leisure horses and ponies in New Zealand. The New Zealand Pony Clubs Association (NZPCA) was chosen as a focus group because it is the only organisation in New Zealand that has registered members who keep horses and ponies for leisure riding and companionship.

Chapter 3 reports on the dietary management and health of horses and ponies ridden at Pony Clubs across New Zealand. The study utilised an online survey tool to distribute a questionnaire, and collected data on the feeding and management practices and the frequency of nutrition-related disorders occurring in the population of horses and ponies maintained on typical New Zealand pasture.

Supplementary information on the data collection methods such as the online survey questionnaire, invitation letter, media coverage and some additional findings are presented in the appendix for this chapter (Appendix A).

The key findings of the study were presented at the annual conference of the New Zealand Society of Animal Production (www.nzsap.org) in Napier on 30th June 2014, during the young members' award session, and the manuscript was published in the conference proceedings.

Fernandes, K.A., Rogers, C.W., Gee, E.K., Bolwell, C.F. and Thomas, D.G. (2014). A cross-sectional survey of rider and horse demographics, and the feeding, health and management of Pony Club horses in New Zealand. *Proceedings of the New Zealand Society of Animal Production*, Napier, New Zealand. **74:** 11-16

CHAPTER 3: A CROSS-SECTIONAL SURVEY OF RIDER AND HORSE DEMOGRAPHICS, AND THE FEEDING, HEALTH AND MANAGEMENT OF PONY CLUB HORSES IN NEW ZEALAND

3.1 Abstract

A cross-sectional survey was conducted to describe rider and horse demographics, and the feeding, health, and management of horses and ponies involved with Pony Clubs in New Zealand. An online survey collected information from members of the New Zealand Pony Clubs Association between 1st November 2012 and 31st January 2013. A total of 502 respondents completed the survey. Most riders were female (95%; 455/481) and were ≤16 years of age (74%; 357/481). Decisions on feeding and management of horses were rarely made by the rider alone (26%; 124/476). The rider's parent or person responsible for the horse were often included in the decision-making, which occurred consistently across all age-groups ≤16 years (P<0.001). Over half (53%; 252/475) of respondents' families had more than 20 years of equine experience. On a scale of 1-9, the median body condition score (BCS) of the nominated Pony Club horses was six (interquartile range 5-6), and only 22% (98/446) of respondents indicated that their horse was overweight (BCS ≥7/9). More than 80% of horses were managed on pasture throughout the year, with 24-hour access to grazing each day. In addition to pasture, 82% (409/502) of respondents fed their horses a combination of commercially available equine feeds, including premixed feeds, cereals, and conserved forages. Overall, owners of Pony Club horses reported few health issues associated with their horse.

3.2 Introduction

The non-racing equine population in New Zealand consists of sport horses (used for the Olympic disciplines of dressage, eventing and show jumping), and recreational horses (horses and ponies primarily used for recreational activities, pleasure riding and companionship). However, the two groups may not be mutually exclusive, as horses may be used for multiple purposes (e.g. the Pony Club horse). Within the non-racing sector, previous research has focused on examining the production and reasons for wastage of sport horses (Rogers and Firth, 2005), and relatively little attention has been paid to recreational horse populations (Rosanowski *et al.*, 2012b).

The New Zealand Pony Clubs Association (NZPCA) is an organisation that promotes horse riding and companionship, and educates young people to develop horsemanship skills. It offers competitions across a wide range of disciplines, providing experience before entry into registered equestrian sport. Currently, the NZPCA is the largest junior equestrian organization with ~9,000 registered members across New Zealand (Anonymous, 2012).

In New Zealand, most horses are managed on pasture all year round, which provides a unique set of management challenges when compared to horses managed intensively in stables (Rogers *et al.*, 2007). In Pony Club- and recreational horse-populations, surveys have identified concerns with the nutritional management of these horses and the prevalence of obesity and behavioural problems (Buckley *et al.*, 2013, Ireland *et al.*, 2013). Hoffmann *et al.* (2009) reported that many recreation-horse owners in the USA lacked knowledge on equine nutrition. The level of nutritional knowledge within recreation-horse owners in New Zealand, and who makes the feeding and management decisions for the horses and ponies ridden by children within the Pony Club association, is currently unknown.

The objective of the study was to conduct a cross-sectional survey to describe the demographic characteristics of the NZPCA population of riders and their horses, describe the general feeding and management of the horses and ponies ridden by members of Pony Club (PC horses), and investigate the owner-reported prevalence of nutrition-related health conditions.

3.3 Materials and Methods

The target population for the survey was riding members of the NZPCA (~5090, Anonymous 2012). The survey was designed and implemented online using the Qualtrics™ Survey Research Software (Qualtrics 2012, Utah, USA), and consisted of 50 open- and close-ended questions examining rider demographics, equestrian experience and the health and management of PC horses. Riders ≥16 years of age and the parents of riders <16 years of age were eligible to participate in the survey. Information was collected about the rider and a nominated PC horse. Parents with multiple children at Pony Club were requested to complete a separate survey for each child, with their individually nominated PC horse (Appendix A-1 to A-3).

Data were collected over a period of three months from 1st November 2012 to 31st January 2013. The survey was advertised via an article containing a hyperlink to the questionnaire in the November 2012 NZPCA e-newsletter, which was also placed on the NZPCA website (www.nzpca.org.nz). Emails were sent via the 16 NZPCA regional administrators and a social network page was created on Facebook (www.facebook.com) to further publicise the study. Three reminders (at 30, 60 days and one week before closing) were sent via e-mail and social media, and eligibility for a prize draw was included at the end of the survey to encourage participation (Appendix A-4 to A-9).

Data were extracted from the software and were examined for inconsistencies and outliers via exploratory data analysis (MS Excel 2010, Microsoft Corporation, USA). Lifetime, adult, and casual members, and respondents that did not complete the first five questions were considered ineligible responses, and were excluded from the dataset. The body condition score (BCS) of the nominated PC horse was reported with reference to a standard 9-point-scale chart that was provided in the survey, and a BCS ≥7 was considered overweight. Commercially available premixed-feed varieties were categorised into low-performance, leisure and maintenance feeds, or high-performance and growth feeds, based on the digestible energy (DE) reported by the feed manufacturer.

The data were analysed in STATA version 12.1 (Stata Corp, College Station, Texas), and simple descriptive statistics are presented for parametric and non-parametric data. Categorical data are presented as count and percentage. The denominator for categorical variables was determined on a per-question basis, and missing values were due to "non-

response" or "no" to the question. As respondents were given the option to select multiple answers within a question, the total percentage for certain categorical variables does not add-up to 100%. The significance of associations between the variables "Decisions on feeding and management of PC horses" and "rider age-groups" was assessed using Chi-squared tests.

3.4 Results

A total of 699 respondents attempted the online survey. After removal of ineligible and incomplete responses, 502 responses were included in the analysis, indicating a completion rate of 72%. Responses were obtained from all 16 NZPCA regions across New Zealand. The majority of responses (62%; 312/502) were from the parents or guardians of Pony Club riders that were <16 years of age.

Most riders were female (95%; 455/481), and had been members of Pony Club for >2 years (Table 3.1). The majority of riders (57%; 273/481) had either no certificate or had low-level (< C level) riding certificates. Decisions on feeding and management of horses were rarely made by the rider alone (Table 3.1). The rider's parent, or person responsible for the horse, often made the management decisions or were included in the decision-making, which occurred consistently across all age-groups ≤16 years (P<0.001). Few respondents (7%; 32/475) indicated that they (or their families) had <2 years of equine management experience, with just over half indicating >20 years of experience.

The median number of horses owned by a Pony Club member was three (interquartile range [IQR] 2-4). Of the nominated horses included in the survey, most were owned for ≤3 years (Table 3.1), 56% (262/468) of which were classified as ponies (≤148 cm wither height). The median age of horses was 12 years (IQR 8-15), just over half were geldings (56%; 264/468) and there were 23 descriptors provided for breed; 50% (235/468) of which were categorised as mixed-breed horses and ponies. On a scale of 1-9, the median BCS was six (IQR 5-6), and only a few respondents reported that their horse was overweight (22%; 96/440).

Table 3.1. Demographic data of riders obtained by an online survey of members of the New Zealand Pony Clubs Association.

Variable	Proportion of respondents % (n)
Geographical region (N=502)	
Upper North Island	50% (253)
Lower North Island	28% (141)
South Island	22% (108)
Period of Membership at Pony Club (N=477)	
< 2 years	8% (36)
2-4 years	29% (137)
5-7 years	28% (135)
8-10 years	21% (98)
>10 years	15% (71)
Rider's age (N=481)	
≤10 years	21% (101)
11-13 years	23% (110)
14-16 years	30% (146)
≥17 years	26% (124)
Highest riding certificate attained (N=481)	
None	22% (104)
D or D+	35% (169)
C or C+	32% (154)
B, A or H	11% (54)
Decisions on feeding and management (N=476)	
By rider alone	26% (124)
By parent or person responsible for the horse	45% (213)
By rider and parent or person responsible for the horse	29% (139)
Respondent or family equine management experience (N=475)	
<2 years	7% (32)
2-10 years	26% (122)
11-20 years	15% (69)
>20 years	53% (252)
Period of ownership of the nominated horse (N=453)	
< 1 year	31% (139)
1-3 years	47% (214)
4-6 years	13% (59)
7-9 years	5% (24)
≥ 10 years	4% (17)

The majority of horses were ridden (schooling or hacking) ≥3 times per week (82%; 370/451), for a median duration of 60 (IQR 45-60) minutes per session. In addition, PC horses were involved in a number of activities and or competitions; the most common were Pony Club rallies (87%; 390/448), dressage (59%; 264/448) and show jumping (57%; 254/448) (Appendix A-10).

The majority of horses were kept on the owner's property (69%; 322/468) or on a lease block (31%; 146/468). Most horses (85%; 425/502) were managed at pasture (Table 3.2). The median paddock size was 0.81 hectares (IQR 0.40-1.42) with most horses kept in groups of three (IQR 2-4) horses. At least 80% (337/420) of the horses had 24-hour access to grazing pasture all year round (Appendix A-11). The remaining group had <6 hours of access to pasture (27%; 22/83), spent approximately 12 hours on pasture and12 hours stabled or yarded (55%; 46/83), or were stabled or yarded for most of the day (18%; 15/83). Pasture quality was commonly managed by cross grazing with sheep or cattle and or topping. At least half of the respondents picked-up horse manure from the paddock and or harrowed as a parasite control measure (Table 3.2).

In addition to pasture, most respondents (82%; 409/502) fed their horses supplementary feeds (Appendix A-12). A minority of respondents (n=143) fed cereal grains, while the majority fed a variety of premixed feeds (n=322) and conserved forages (n=402), either alone or in combination with other feeds (Table 3.2). The majority of respondents (n=249) fed premixed feeds marketed for low performance, leisure and maintenance (n=32 varieties, mean DE12.1 MJ/kg DM [8.5-14.0]) and 173 respondents fed premixed feeds marketed for medium-to-high performance and growth (n=20 varieties, mean DE 13.97 MJ/kg DM [12.0-17.8]). Thirty-five per cent (18/52) of these premix varieties were marketed as "cool feeds" (mean DE 12.8 MJ/kg DM [11-17.8]) and were fed by 77% (247/322) of the respondents. Most respondents (82%; 410/502) fed at least one type of supplement, the most commonly used was a mineral and vitamin mix (63%; 260/410) (Appendix A-13 and A-14).

Table 3.2. Management of pasture and feeding practices of Pony Club horses obtained by an online survey of members of the New Zealand Pony Clubs Association.

Variable	Proportion of respondents % (n)
Pasture Management	
Type of grazing provided to the nominated horse (N=426)[1]	
Set stocked	14% (59)
Rotation	73% (312)
Strip	54% (228)
Type of pasture provided to the nominated horse (N=415)	
Dairy	17% (69)
Sheep and beef	35% (144)
Horse	23% (94)
Other	11% (47)
Don't know	15% (61)
Pasture management (N=414)[1]	
Cross graze	64% (264)
Pick-up horse manure	59% (246)
Harrow	50% (209)
Topping	42% (172)
Supplementary feeds	82% (409/502)
Cereal grains[1]	35% (143/409)
Barley	73% (105)
Oats	30% (43)
Maize	6% (9)
Other grain and grain-by-products	22% (32)
Other fibre sources[1]	29% (119/409)
Sugar beet	58% (83)
Copra	25% (36)
Commercial premixed feeds[1]	79% (322/409)
Low performance, leisure and maintenance	77% (249)
Medium-to-high performance and growth	54% (173)
Conserved forages	98% (402/409)
Chaff[1]	72% (294/409)
Lucerne	52% (154)
Meadow	34% (99)
Oat and oat-straw	27% (78)
Lucerne and meadow mix	14% (40)
Other chaff	6% (18)
Hay[1]	84% (345/409)
Meadow	92% (317)
Lucerne	13% (43)
Other hay	10% (33)
Fermented forages	50% (206/ 409)
Commercial haylage	79% (163)
Locally-made haylage	21% (43)

[1] A respondent could have selected multiple options in a sub-category

Less than half of the respondents knew their horse's body weight (45%; 210/462), and of those that provided a weight, half had guessed the horse's weight (51%; 108/210), 39% (81/210) used a weight tape, and only 10% (21/210) had weighed their horse on a set of scales. Most horses were shod (67%, 248/372). Horses were trimmed or shod a median number of eight times per year (IQR 6-10), which was typically done by a farrier (78%; 123/157 and 94%; 112/119, respectively).

Anthelmintics were administered to most horses (99%; 421/423) at a median of four (IQR 3-5) times per year and half (55%; 183/331) of the horses were vaccinated (Table 3.3). Most (90%; 358/397) horses received a dental examination at least once a year, which was more commonly performed by an equine dentist rather than a veterinarian. Less than half (44%; 211/485) of the respondents identified a health issue associated with their PC horse. The conditions reported ranged from obesity, colic, laminitis, grass staggers, and gastric ulcers, to a variety of injuries and minor conditions (Table 3.3). A veterinarian was reported to have examined most cases of colic (73%; 19/26) and gastric ulcers (67%; 12/18), but the majority of the reported cases of obesity, laminitis, and grass staggers, had not been examined by a veterinarian.

Table 3.3. Preventive care and owner-reported health issues of Pony Club horses obtained by an online survey of members of the New Zealand Pony Clubs Association.

Variable	Proportion of respondents % (n)	
Preventive health care		
Anthelmintics	99% (421/423)	
Vaccinations [1]	55% (183/331)	
Tetanus alone		21% (39)
Strangles alone		<1% (3)
Both tetanus and strangles		23% (42)
Did not specify the type of vaccine		54% (99)
Dental examinations	90% (358/397)	
By equine dentist		68% (111)
By veterinarian		30% (49)
Owner-reported health issues[1]	44% (211/485)	
Obesity		14% (29)
Colic		12% (26)
Laminitis		12% (26)
Grass staggers		12% (25)
Gastric ulcers		9% (18)
Metabolic and endocrine syndromes		6% (12)
Other (injury and minor) conditions		36% (75)

[1] A respondent could have selected multiple options in a sub-category

3.5 Discussion

This study aimed to describe the rider and horse demographics, and the feeding, health, and management of PC horses in New Zealand. The survey responses appeared to reflect the population spread, and the previously estimated demographics of Pony Club participants across New Zealand (Anonymous, 2012). However, the results of the study may present some limitations due to recall and non-response bias.

Many of the parents had considerable (>20 years) equine experience and were actively involved in the decisions made on the management of PC horses. This is similar to the findings of Buckley *et al.* (2004) in Australia, and indicates that many parents in our survey had significant prior equine experience, before their children became involved in Pony Club. In contrast, a poor understanding of equine nutrition and management was reported in a survey of horse owners within the USA (Hoffman *et al.*, 2009).

The majority of horses were managed continuously on pasture throughout the year, and there was no seasonal variation in the hours of grazing pasture. This finding is in contrast to Hotchkiss *et al.* (2007), and may be a reflection of the temperate climate and production use of PC horses in New Zealand (Hoskin and Gee, 2004). The horses with restricted access to grazing pasture were mostly provided 12:12 hour-rotations, which was similar to that reported in the United Kingdom (Wylie *et al.*, 2013), and possibly reflects ease of management for the parents, given that most horses were managed at the home property.

From the data collected, we were unable to quantify the pasture dry matter (DM) available to, or consumed by the horses. The inability to quantify feed intake is a difficulty with pasture management of horses (Hoskin and Gee, 2004). This may be a reason for the large number of respondents that provided supplementary (grain or premixed) feeds in addition to pasture. In commercial equine production systems, supplementary feeds are usually provided at a set quantity and the remaining daily energy requirement is met with pasture (Rogers *et al.*, 2007). Within the Pony Club population, the provision of supplementary feeds were at much lower quantities, in comparison to that fed for growth and performance in production systems; but appeared to be offered due to a similar motivation to "balance the ration". A number of the premixed feeds fed to PC horses in our study were marketed as "cool feeds", which had a DE content similar to that of medium-to-high-performance and growth feeds. This choice of feeding may reflect the perception of the respondents that these feeds

provided additional DE without the associated "heating" effects of some traditional feeds such as oats or racehorse premixes.

Most horses were ridden ≥3 times a week for an average duration of 60 minutes, and were actively involved in Pony Club rallies and other competitions. This level of activity may explain the reason why only a few horses had higher BCS (≥7/9). It is likely that the owners actively tried to maintain an ideal BCS (perceived) and used BCS as a measure of horse health (Buckley *et al.*, 2004).

A study in the United Kingdom reported that 32% of horses and ponies had a long-term or recurrent health condition (Ireland *et al.*, 2013). In contrast, few health issues were reported in our study. A primary reason for this could be the management of horses on pasture, which appears to be associated with a lower prevalence of health issues such as colic and respiratory conditions that commonly occur in stabled horses (Perkins *et al.*, 2005). Alternatively, it may be that the health issues in the current survey were under-reported, as owner-reported prevalence may be subject to recall bias, and has been shown to differ to that reported by a veterinarian (Ireland *et al.*, 2011).

The frequency of providing regular preventive health-care such as dental examinations, hoof care, and the administration of anthelmintics, was high in the present survey, which was similar to that reported for non-commercial horses in New Zealand (Rosanowski *et al.*, 2012a). However, the level of vaccination use was low by international standards (Ireland *et al.*, 2013). This low vaccination-rate possibly relates to the low prevalence of infectious equine diseases within New Zealand (Anonymous, 1989).

The study has provided baseline information on the demographics and management of PC horses in New Zealand. In contrast to similar horse populations in other parts of the world, the results of the present survey showed that horses used at Pony Clubs in New Zealand were proactively managed on pasture by experienced owners.

3.6 Acknowledgements

The authors thank the NZPCA for their cooperation and participation, and Horsetalk NZ for promoting the online survey. We also thank the equine companies (Fiber Fresh Feeds, Speciality Seeds, Horse Gifts and NZ Horse and Pony) that kindly sponsored the prizes. The first author (KAF) was financially supported by the Ministry of Business, Innovation and Employment and a Massey University Doctoral Scholarship.

Appendix A

This appendix provides supplementary information on the survey design and data collection methods and some results presented in Chapter 3.

Survey design and data collection methods

A-1. Copy of the online survey questionnaire.

A-2. Copy of the body condition score chart.

A-3. Regional distribution of the estimated number of Pony Club members.

A-4. Copy of the invitation email sent to Pony Club members.

A-5. Copy of the reminder email sent to Pony Club members.

A-6. Excerpt from the newsletter circulated to the Pony Clubs members.

A-7. Excerpt from the featured news on the New Zealand Pony Clubs Association website.

A-8. Excerpt from the Facebook page designed for the survey.

A-9. Excerpt from the article published in Horsetalk news.

Additional Results

A-10. Participation of riders and the nominated Pony Club horse in various activities and competitions.

A-11. Seasonal grazing turnout for Pony Club horses.

A-12. Varieties of commercial horse feeds offered to Pony Club horses.

A-13. Categorisation and digestible energy of commercially available premixed feeds offered to Pony Club horses.

A-14. Supplements fed to Pony Club horses.

A-1. A copy of the online survey questionnaire designed using the Qualtrics online survey software and insight platform (www.qualtrics.com)

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

THE NZPCA EQUINE HEALTH AND MANAGEMENT SURVEY

INFORMATION SHEET

Welcome to 'The NZPCA Equine Health and Management Survey'

Click the "NEXT" button to continue.

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Dear NZPCA Member,

Thank you for choosing to participate in 'The NZPCA Equine Health and Management Survey'.

The Equine Team at Massey University is passionate about improving the health of our equine companions. In this regard, we would like to explore further into issues relating to the health and management of 'non-racing' horses and ponies in New Zealand.

This survey is designed to give you an opportunity to tell us about the feeding, management and health care of your horse / pony, which is primarily being used for recreational riding and/or at Pony Club activities.

General Instructions:

1. All responses to the survey will be anonymous; any personal information will remain strictly confidential.
2. Pony club riders must be 16 years of age or older to participate in the survey.
3. Riders under the age of 16 years will require parents to take the survey on their behalf.
4. Parents with more than one child at Pony Club are welcome to take the survey multiple times for each rider and their horse/pony.
5. The survey will only take about 10-15 minutes to complete.
6. The survey link will remain active until January 2013.
7. Please contact the researcher for any assistance or queries at the e-mail address below.

A summary of results will be available via the New Zealand Pony Club Association.

Your assistance is much appreciated.

Kind regards,

Karlette Fernandes
PhD Researcher

Contact: K.A.Fernandes@massey.ac.nz

This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researchers named below are responsible for the ethical conduct of this research.
If you have any concerns about the conduct of this research that you wish to raise with someone other than the researchers, please contact Professor John O'Neill, Director, Research Ethics, telephone +64 06 350 5249, email humanethics@massey.ac.nz

Te Kunenga ki Pūrehuroa

Institute of Food, Nutrition and Human Health
Private Bag 11222, Palmerston North 4442, New Zealand T 64 6 356 4726 F 64 6 350 5657 http://fnhh.massey.ac.nz

Print

Click the "NEXT" button to continue.

RESPONDENT DETAILS

Are you a member of the New Zealand Pony Club Association (NZPCA)?

- *Yes*
- *No*

This survey is designed to capture equine health and management information from riders or their parents, who are members of the New Zealand Pony Club Association.

Since you have selected 'No', the questions in this survey may not pertain to your interests.

You are welcome to contact the researcher at K.A.Fernandes@massey.ac.nz for further information.

Thank you for your time.

You are _____

- *The rider at pony club (you must be 16 years of age or older)*
- *A parent / guardian / person responsible for a horse or pony (for riders under 16 years of age)*
- *Other members (Technical, casual or life members)*

This survey is designed to capture equine health and management information from riding members (or their parents) at the New Zealand Pony Club Association.

Since you have selected 'Other' type of membership, the questions in this survey are not applicable to you.

You are welcome to contact the researcher at K.A.Fernandes@massey.ac.nz for further information.

Thank you for your time.

Rider details

Where is your Pony Club located?

How long have you been a member of Pony Club?

What is your age and gender?

Age
Gender

What certificate level do you currently hold?

None D D+ C C+ B A Riding A H

In general, how many years of experience does your family have with horses?

Who makes 'decisions' on the feeding, exercise and management of your horse / pony? (More info on 'decisions')

- Pony club rider
- Parent of the rider
- Person responsible for horse / pony
- Other (Please specify)

Parent_Rider details

Where is your Pony Club located?

How many of your children ride at Pony Club?

	0	1	2	3	4	5	6	7	8
Children									0

For the purpose of this survey, please randomly choose one child.

(NOTE: We'd appreciate if you could take this survey again for another child)

Click the "Next" button to continue

How long has this child (the rider) been a member of Pony Club?

What is the rider's age and gender?

Age
Gender

What certificate level does the rider currently hold?

None D D+ C C+ B A Riding A H

In general, how many years of experience do you or your family have with horses?

Who makes 'decisions' on the feeding, exercise and management of your horse / pony? (More info on 'decisions')

- Pony club rider
- Parent of the rider
- Person responsible for horse / pony
- Other (Please specify)

Other members

In general, how many years of experience do you or your family have with horses?

Who makes 'decisions' on the feeding, exercise and management of your horse / pony? (More info on 'decisions')

- Pony club rider
- Parent of the rider
- Person responsible for horse / pony
- Other (Please specify)

HORSE DETAILS

How many horses / ponies do you currently own or care for?

	0	1	2	3	4	5	6	7	8	9	10	Not Applicable
Horses												
Ponies												

For the purpose of this survey, choose one horse or pony.

(NOTE: If you are the parent of a rider, please choose a horse/ pony associated with this rider)

What is the name of this horse / pony?

Please type name here

How long have you owned "${q://QID25/ChoiceTextEntryValue}"?

Where is "${q://QID25/ChoiceTextEntryValue}" usually located?

- At home property
- Leased property away from home
- Other (Please specify)

How old is "${q://QID25/ChoiceTextEntryValue}"?

What is "${q://QID25/ChoiceTextEntryValue}'s" gender?

- Mare
- Gelding

What best describes "${q://QID25/ChoiceTextEntryValue}'s" breed?

Horse

- Thoroughbred
- Thoroughbred cross
- Standardbred
- Stationbred
- Sport horse
- Warmblood
- Kaimanawa
- Arab
- Arab cross
- Anglo-Arab
- Morgan
- Mixed breed

Pony

- Welsh
- Welsh cross
- Connemara
- Timor
- Timor cross
- Shetland cross
- Riding pony
- Mixed breed

- Other (Please specify)

What is "${q://QID25/ChoiceTextEntryValue}'s" height?
(Please type a value in either centimeters or hands)

- Centimeters
- Hands
- Don't know

How much does "${q://QID25/ChoiceTextEntryValue}" weigh?

- Kilograms
- Don't know

By which method was it weighed?

- Weigh scale
- Weigh tape
- A guess / estimate
- Other (Please specify)

On a 1-9 scale, which picture best describes "${q://QID25/ChoiceTextEntryValue}'s" body condition score?
(Click on the Chart or each score to view an illustrated example)

Equine Body Condition Score Chart

- Score 1
- Score 2
- Score 3
- Score 4
- Score 5
- Score 6
- Score 7
- Score 8
- Score 9
- Don't know

How often is "${q://QID25/ChoiceTextEntryValue}" ridden?

- More than 5 times a week
- 3-4 times a week
- Once or twice a week
- Once a fortnight
- Once a month
- Less than once a month
- Never
- Other (Please specify)

Approximately how long are the riding sessions each time?
(Please type a value in either minutes or hours)

- Minutes
- Hours

In the last 12 months, how often has "${q://QID25/ChoiceTextEntryValue}" been involved in each of the following competitions?

	Never	Once a Year	Once in 6 months	Once in 3 months	Once a month	Once a fortnight	Once a week
Competitive Trial Riding (CTR)	○	○	○	○	○	○	○
Dressage	○	○	○	○	○	○	○
Endurance	○	○	○	○	○	○	○
Games	○	○	○	○	○	○	○
Horse Trials / Eventing	○	○	○	○	○	○	○
Pony Club Rally	○	○	○	○	○	○	○
Showing	○	○	○	○	○	○	○
Show Hunter	○	○	○	○	○	○	○
Show Jumping	○	○	○	○	○	○	○
Other (Please specify) ☐	○	○	○	○	○	○	○

If you would like to tell us more about ${q://QID25/ChoiceTextEntryValue} participation in equine competitions, please comment here.

☐

HORSE HEALTH INFORMATION

In the last 12 months, how often did you consult the following people / sources for advice on "${q://QID25/ChoiceTextEntryValue}'s" HEALTH?
(Please type in a value for number of times per year)

	Number of times per year (0 = not consulted)
Decide yourself (e.g. read books, Internet, etc.)	☐
Equine nutritionist	☐
Equine store / feed supplier	☐
Farrier	☐
Feed Manufacturer	☐
Friend	☐
Horse trainer	☐
Person caring for the horse / pony	☐
Riding instructor	☐
Veterinarian	☐
Other (Please specify) ☐	☐

In the last 12 months, did "${q://QID25/ChoiceTextEntryValue}" get the following treatments?
(Please type in a value for number of times per year)

	Number of times per year (0 = not treated)
Vaccination	☐
De-worming	☐
Dental Check	☐
Hoof trimming	☐
Shoeing	☐
Other treatments (Please specify) ☐	☐

What was it vaccinated against?

- ☐ Tetanus
- ☐ Strangles
- ☐ Other (Please specify)
  ☐

By whom was the dental done?

- ○ Veterinarian
- ○ Equine Dentist
- ○ Other (Please specify)
  ☐

Who did the hoof trimming?

- ☐ Farrier
- ☐ Parent
- ☐ Rider
- ☐ Person responsible for the horse / pony
- ☐ Other (Please specify)
  ☐

Who shod the horse / pony?

- ☐ Farrier
- ☐ Parent
- ☐ Rider
- ☐ Person responsible for the horse / pony
- ☐ Other (Please specify)
  ☐

Ever since you've known "${q://QID25/ChoiceTextEntryValue}", did it have any of the following conditions?

- ☐ Colic
- ☐ Cushing's disease
- ☐ Gastric Ulcers
- ☐ Grass Staggers
- ☐ Insulin resistance
- ☐ Laminitis / founder
- ☐ Metabolic syndrome
- ☐ Obesity
- ☐ Tying-up
- ☐ Other (Please specify) ☐
- ☐ None of the above

In the last 12 months, did ${q://QID25/ChoiceTextEntryValue} have any of these conditions?

	Condition occurred		In which season?				Examined by a Vet?		Current status?		
	Yes	No	Spring	Summer	Autumn	Winter	Yes	No	Worse	Same	Improved
» Colic	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Cushing's disease	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Gastric Ulcers	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Grass Staggers	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Insulin resistance	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Laminitis / founder	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Metabolic syndrome	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Obesity	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Tying-up	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» Other (Please specify)	○	◉	☐	☐	☐	☐	○	◉	○	○	○
» None of the above	○	◉	☐	☐	☐	☐	○	◉	○	○	○

In the last 12 months, have you noticed any of the following signs with "${q://QID25/ChoiceTextEntryValue}"?

BEHAVIOUR

- ☐ *Crib biting*
- ☐ *Fence walking / pacing in the stable*
- ☐ *Pawing & looking at flanks*
- ☐ *Weaving side-to-side*
- ☐ *Wind sucking*

HEALTH / APPETITE

- ☐ *Depression or lethargy*
- ☐ *Dental pain*
- ☐ *Increased appetite*
- ☐ *Increased drinking*
- ☐ *Increased urination*
- ☐ *Reduced appetite*
- ☐ *Reduced drinking*
- ☐ *Reduced urination*

BODY CONDITION

- ☐ *Fat deposition around neck (cresty neck appearance)*
- ☐ *Long curly hair coat even in summer*
- ☐ *Weight gain*
- ☐ *Weight loss*

HOOF & GAIT

- ☐ *Long, curved hooves*
- ☐ *Rings on hoof walls*
- ☐ *Reluctance to walk / trot*
- ☐ *Sore feet / pain in hooves*
- ☐ *Standing with forelegs stretched out*
- ☐ *Uneven / irregular gait*

Other / None

- ☐ *Other (Please specify)*
- ☐ *None of the above*

Was "${q://QID25/ChoiceTextEntryValue}" examined for these signs?

	Examined by a Vet?		Current Status		
	Yes	No	Worse	Same	Improved
» Crib biting	○	◉	○	○	○
» Fence walking / pacing in the stable	○	◉	○	○	○
» Pawing & looking at flanks	○	◉	○	○	○
» Weaving side-to-side	○	◉	○	○	○
» Wind sucking	○	◉	○	○	○
» Depression or lethargy	○	◉	○	○	○
» Dental pain	○	◉	○	○	○
» Increased appetite	○	◉	○	○	○
» Increased drinking	○	◉	○	○	○
» Increased urination	○	◉	○	○	○
» Reduced appetite	○	◉	○	○	○
» Reduced drinking	○	◉	○	○	○
» Reduced urination	○	◉	○	○	○
» Fat deposition around neck (cresty neck appearance)	○	◉	○	○	○
» Long curly hair coat even in summer	○	◉	○	○	○
» Weight gain	○	◉	○	○	○
» Weight loss	○	◉	○	○	○
» Long, curved hooves	○	◉	○	○	○
» Rings on hoof walls	○	◉	○	○	○
» Reluctance to walk / trot	○	◉	○	○	○
» Sore feet / pain in hooves	○	◉	○	○	○
» Standing with forelegs stretched out	○	◉	○	○	○
» Uneven / irregular gait	○	◉	○	○	○
» Other (Please specify)	○	◉	○	○	○
» None of the above	○	◉	○	○	○

If you would like to share any more health related information about ${q://QID25/ChoiceTextEntryValue}, please comment here.

HORSE FEEDING MANAGEMENT

In the last 12 months, how often did you consult the following people / sources regarding "${q://QID25/ChoiceTextEntryValue}'s" NUTRITIONAL needs?
(Please type a numerical value for number of times per year)

	Number of times per year (0 = not consulted)
Decide yourself (e.g. read books, Internet, etc.)	
Equine nutritionist	
Equine store / feed supplier	
Farrier	
Feed Manufacturer	
Friend	
Horse trainer	
Person caring for the horse / pony	
Riding instructor	
Veterinarian	
Other (Please Specify)	

Where is "${q://QID25/ChoiceTextEntryValue}" kept during each season of the year?

(Please choose an option or enter the number of hours per day in paddock and stable /yard for each season)

	24 hours per day Paddock	Hours per day Paddock	Hours per day Stable / yard	24 hours per day Stable / yard
Spring (Sept - Nov)				
Summer (Dec - Feb)				
Autumn (Mar - May)				
Winter (Jun - Aug)				

What type of stocking/ grazing system do you follow?

- Set stocking (Fixed paddock)
- Rotational grazing (Multiple paddocks)
- Strip grazing or break fencing (Temporary divided paddock)
- Not applicable - horse / pony is stabled always
- Other (Please specify)

How is "${q://QID25/ChoiceTextEntryValue}" stocked?

- Individually
- In a group of

How many paddocks are available for ${q://QID25/ChoiceTextEntryValue} to graze on?

	0	1	2	3	4	5	6	7	8	9	10	Not Applicable
Number of paddocks available												

What is the average size of the grazing paddocks?
(Please type in either hectares or acres)

- Hectares
- Acres

What best describes the type of pasture available?

- Dairy type of pasture
- Sheep & beef type of pasture
- Horse specific type of pasture
- Other (Please specify)
- Don't know

Could you specify the type of pasture grass-mix sown in the paddock?

- Don't know

Grasses

- Browntop
- Cocksfoot (Orchard Grass)
- Couch Grass
- Fine Fescue
- Kikuyu
- Lotus
- Meadow Foxtail
- Perennial Ryegrass
- Phalaris
- Prairie Grass
- Tall Fescue

Legumes

- Red Clover
- White Clover

- Other grasses / legumes or herbs sown in the paddock (e.g. plantain, chicory, etc.)

(If you would like to describe your pasture mix, please comment here).

In the last 12 months, what was the approximate height of the grass available in each season?

	Very short grass "Grazed off close to the ground" (Less than 2 cm)	Short grass "Grass the height of a match-stick" (2 - 4 cm)	Medium grass "Enough grass to cover the horses' hooves" (5-8 cm)	Tall grass "Long / lush grass" (more than 8 cm)	Don't know
Spring (Sept - Nov)					
Summer (Dec - Feb)					
Autumn (Mar - May)					
Winter (Jun - Aug)					

What is the approximate percentage of weeds in the grazing paddock?

	Percentage											Not Applicable
	0	5	10	15	20	25	30	35	40	45	50	
Weeds												

Do you practise any of the following pasture management techniques?

	Yes	No
Apply fertilizer	○	◉
Cross grazing with other livestock (e.g. cattle / sheep)	○	◉
Harrow	○	◉
Remove / hand pick dung from pasture	○	◉
Remove weeds manually	○	◉
Re-seeding	○	◉
Weed spraying	○	◉
Topping	○	◉
Other (Please specify)	○	○

What commercial feeds do you give "${q://QID25/ChoiceTextEntryValue}"?
(Please select the name of the feed manufacturer)

- ☐ CopRice / NutriRice
- ☐ CRT - McMillan Equine Feeds
- ☐ Dunstan Horse Feeds
- ☐ Fiber Fresh Feeds
- ☐ Hygain
- ☐ H.R. Fiskens & Sons
- ☐ Mitavite
- ☐ NRM
- ☐ Prydes
- ☐ Other (Please specify the feed manufacturer and name of the feed)
- ☐ Not Applicable - You do not feed commercial diets

Please tell us the brand name of the feed given to "${q://QID25/ChoiceTextEntryValue}".

CopRice / NutriRice
- ☐ Cool Conditioner (Horse & Pony pellets)
- ☐ P'Performance Horse pellets
- ☐ Show & Competition
- ☐ Versatile
- ☐ Veteran
- ☐ Other (Please specify the name)

CRT - McMillan
- ☐ Cool Feed
- ☐ Free-Up
- ☐ Manetane
- ☐ Sport Horse
- ☐ Other (Please specify the name)

Dunstan Horse Feeds
- ☐ All-4-Feet
- ☐ All-you-need
- ☐ Cool Feed
- ☐ Eezy Beet
- ☐ Eezy Mix
- ☐ Fiber Balancer
- ☐ Fiber Grow
- ☐ Fiber Plus
- ☐ Grass Balancer
- ☐ Pasture Plus
- ☐ Sugar Beet
- ☐ Other (Please specify the name)

Fiber Fresh Feeds
- ☐ FiberEdge
- ☐ FiberEzy
- ☐ FiberMix
- ☐ FiberProtect
- ☐ FiberSure

Hygain
- ☐ All Rounder
- ☐ Balanced
- ☐ Equine Senior
- ☐ Honey B
- ☐ Ice
- ☐ Tru Care
- ☐ Zero
- ☐ Other (Please specify the name)

H.R. Fiskens & Sons
- ☐ Horse Balancer
- ☐ Maintenance Mix
- ☐ Over Drive
- ☐ Parole
- ☐ Other (Please specify the name)

Mitavite
- ☐ Cool Performer
- ☐ Economix Active
- ☐ Gumnuts
- ☐ Promita
- ☐ Xtra-Cool
- ☐ Other (Please specify the name)

NRM
- ☐ Coolade
- ☐ Equi Jewel
- ☐ Equine Balancer
- ☐ Horse & Pony
- ☐ Lite Brew
- ☐ Low GI Sport
- ☐ Sweet Feed
- ☐ Other (Please specify the name)

Prydes
- ☐ EasiFeed
- ☐ EasiPerformance
- ☐ EasiResponse
- ☐ EasiRide
- ☐ EasiSport
- ☐ Other (Please specify the name)

What straight feeds / grains do you feed "${q://QID25/ChoiceTextEntryValue}"?

- Whole Oats
- Crushed Oats
- Whole / boiled Barley
- Crushed Barley
- Crushed / kibbled Maize
- Copra Meal
- Bran
- Pollard
- Other (Please specify)
- Not Applicable - You do not feed gains/ straight feeds

What type of chaff / hay / conserved forage do you feed "${q://QID25/ChoiceTextEntryValue}"?

Chaff / forage

- Lucerne chaff
- Lucerne cubes
- Lucerne-Meadow chaff
- Meadow hay chaff
- Oaten chaff
- Oat straw chaff
- Pea vine - Clover chaff
- Timothy chaff

Hay / conserved forage

- Haylage / baleage
- Lucerne hay
- Red clover hay
- Meadow hay
- Pea vine hay

- Other (Please specify)
- Not Applicable - You do not feed chaff / hay / conserved forage

What type of supplements do you feed "${q://QID25/ChoiceTextEntryValue}"?

	Yes	No
Calming Supplements	○	◉
Digestive Supplements	○	◉
Electrolytes	○	◉
Hoof Supplements	○	◉
Joint Supplements	○	◉
Mycotoxin Binders	○	◉
Selenium only	○	◉
Vitamin & Mineral Supplements	○	◉
Other (Please Specify)	○	○

Would you like to share more information about "${q://QID25/ChoiceTextEntryValue}'s" diet / management?.. Please comment here.

Thank You

Thank You! We appreciate your participation in this survey.

Please have a go at our prize draw to WIN
exciting Gift vouchers from our sponsors.

Fill in your contact details below so we can get in touch with you.
We assure you personal information will remain confidential.

First name

Last name

e-mail address

Telephone Number

Acknowledgment:

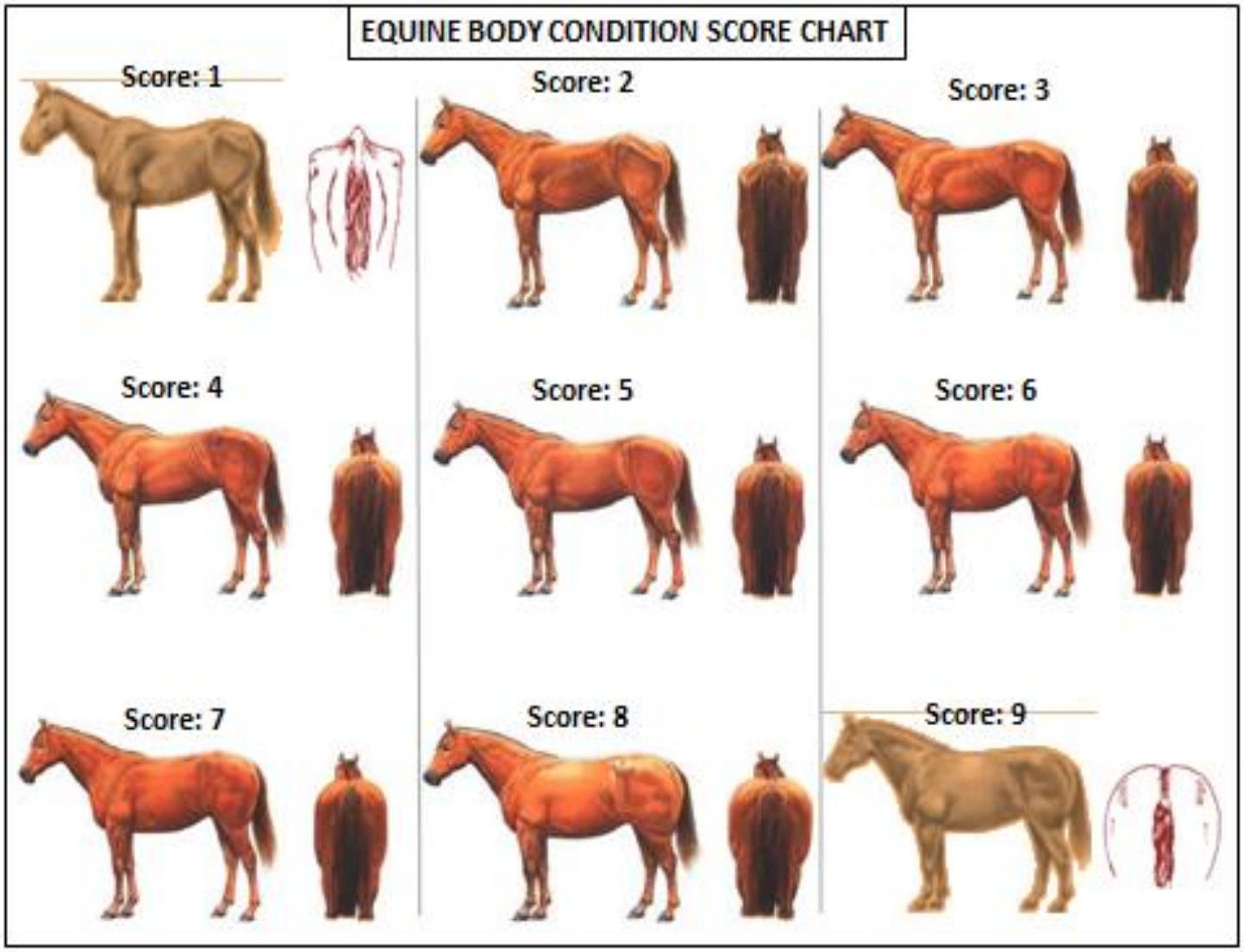
We thank the New Zealand Ministry of Business, Innovation and Employment - Science and Innovation group (MSI) for funding this PhD research project.
We also thank the New Zealand Pony Club Association for for their co-operation.

Ministry of Business, Innovation & Employment | Science + Innovation

THE NEW ZEALAND PONY CLUB NZPCA

* For further details please contact the researcher at K.A.Fernandes@massey.ac.nz

A-2. A copy of the body condition score chart provided to the respondents in the online survey questionnaire.



A-3. Regional distribution of the estimated number of Pony Club members.

No.	Pony Club region	Branches	NZPCA members*	Riding members (estimated 2012-13) Under 18 years	18-25 years	Total	Number of survey respondents
1	Ashburton-South Canterbury-North Otago	9	431	204	24	228	14
2	Auckland	8	689	248	52	300	95
3	Bay of Plenty	17	622	344	31	375	35
4	Canterbury	20	1178	550	60	610	45
5	Franklin Thames Valley	18	691	343	27	370	22
6	Gisborne-Waiora	3	90	52	2	54	2
7	Hawkes Bay	7	520	257	19	276	32
8	King Country	0	66	33	1	34	1
9	Manawatu-West Coast	14	557	271	16	287	59
10	Malborough-Nelson-West Coast	5	396	181	20	201	18
11	Northland	17	483	220	20	240	28
12	Otago-Southland	32	921	457	58	515	31
13	Taranaki	6	460	235	23	258	27
14	Waikato	12	664	361	29	390	24
15	Wairarapa-Wellington	5	378	214	13	227	22
16	Waitemata-Rodney	21	1314	640	85	725	47
	Grand Total	194	9460	4610	480	5090	502

Adapted from NZPCA Annual Report 2013

*Includes life-time, casual, adult and technical members of the New Zealand Pony Clubs Association (NZPCA)

A-4. A copy of the invitation emailed to the 16 regional coordinators of Pony Club branches across New Zealand.

Dear Pony Club riders and parents,

We are conducting a survey on the health and management of horses and ponies ridden at pony clubs in New Zealand.

Your assistance in this survey is greatly appreciated. All responses will be anonymous, even if you enter the prize draw at the end.

A summary of the findings will be distributed through the NZPCA.

Please click on the link below to start the survey, which should take about 10 minutes to complete.

Click here or copy and paste the link in your internet browser:

http://masseybusiness.eu.qualtrics.com/SE/?SID=SV_egH1qPOLbDCTUxv

We are also available on Facebook at:
https://www.facebook.com/Equine.Health.Management

Look forward to your participation.

Many thanks,

Karlette

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND
THE ENGINE OF THE NEW NEW ZEALAND

Karlette Anne Fernandes
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Email K.A.Fernandes@massey.ac.nz
Address Institute of Food, Nutrition and Human Health
Massey University, Private Bag 11222
Palmerston North 4442, New Zealand
Website www.massey.ac.nz

A-5. A copy of the reminder email sent to the 16 regional coordinators of Pony Club branches across New Zealand.

REMINDER to participate in survey: CLICK on the link below

Dear Pony Club riders and parents,

Season's Greetings!

The Equine Health and Management Survey – Pony Clubs New Zealand has been live from November 2012 to January 2013. Our objective is to collect information relating to the health, feeding and general management of horses and ponies involved in Pony Club activities in NZ.

Over the past couple of months, we have received around 300 responses from an estimated 5000 Junior and Senior riding members at Pony Clubs throughout New Zealand.

We encourage all riders/ parents to PARTICIPATE in the ONLINE survey to help us build a better response in order to achieve good results.

Please click on the link below to start the survey or copy and paste the link in your internet browser: http://masseybusiness.eu.qualtrics.com/SE/?SID=SV_egH1qPOLbDCTUxv

Find us on Facebook at https://www.facebook.com/Equine.Health.Management

Looking forward to an enthusiastic response!
Many thanks,
Karlette

(P.S. There are some fantastic prizes to win!)

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UNIVERSITY OF NEW ZEALAND
THE ENGINE OF THE NEW NEW ZEALAND

Karlette Anne Fernandes
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Address Institute of Food, Nutrition and Human Health
Massey University, Private Bag 11222
Palmerston North 4442, New Zealand
Website www.massey.ac.nz

A-6. An excerpt from the New Zealand Pony Clubs Association newsletter emailed to the members in October 2012, to request participation in the online survey.

GIDDY UP

The Official Newsletter of the New Zealand Pony Clubs Association

October, 2012

From Massey University

We are conducting a survey on the health and management of horses and ponies ridden at pony clubs in New Zealand. The online survey asks generic questions relating to the management of ponies and health issues

Your assistance in this survey is greatly appreciated. All responses will be anonymous, even if you enter the prize draw at the end.

A summary of the findings will be distributed through the NZPCA.

Please click on the link below to start the survey, which should take about 10 minutes to complete.

Click here or copy and paste the link in your internet browser:

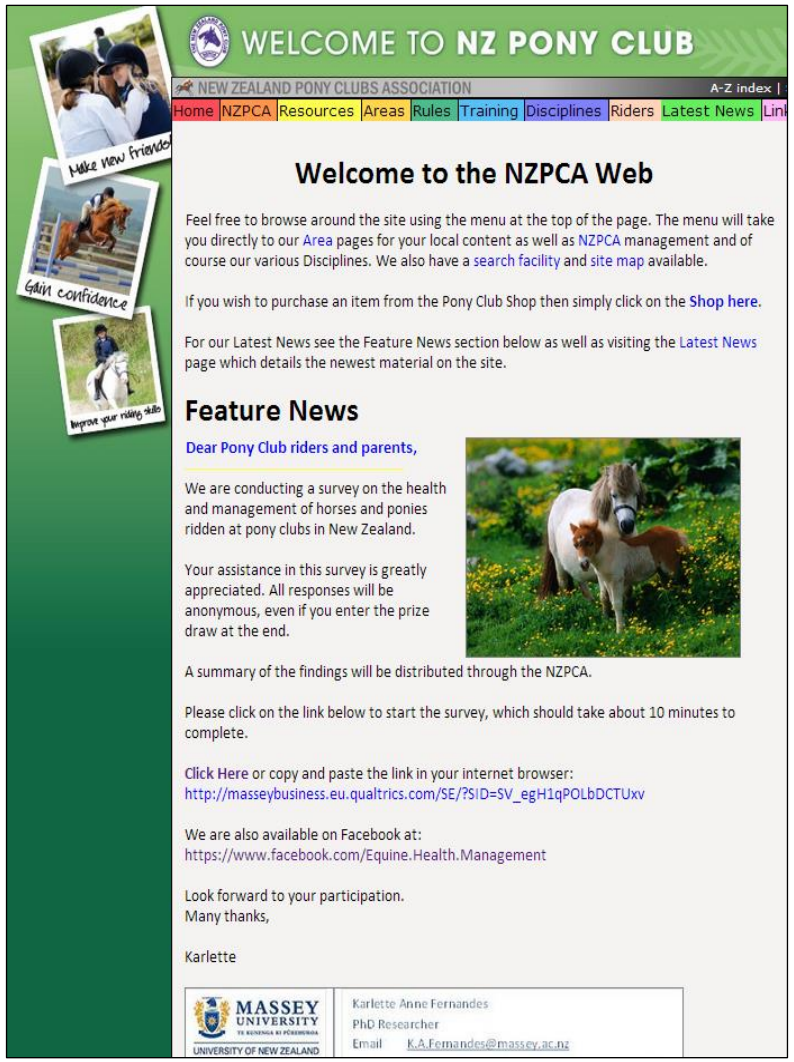
http://masseybusiness.eu.qualtrics.com/SE/?SID=SV_egH1qPOLbDCTUxv

Looking forward to your participation.

Many thanks,

Karlette

A-7. An excerpt from the New Zealand Pony Clubs Association website, advertising the survey in the featured news.

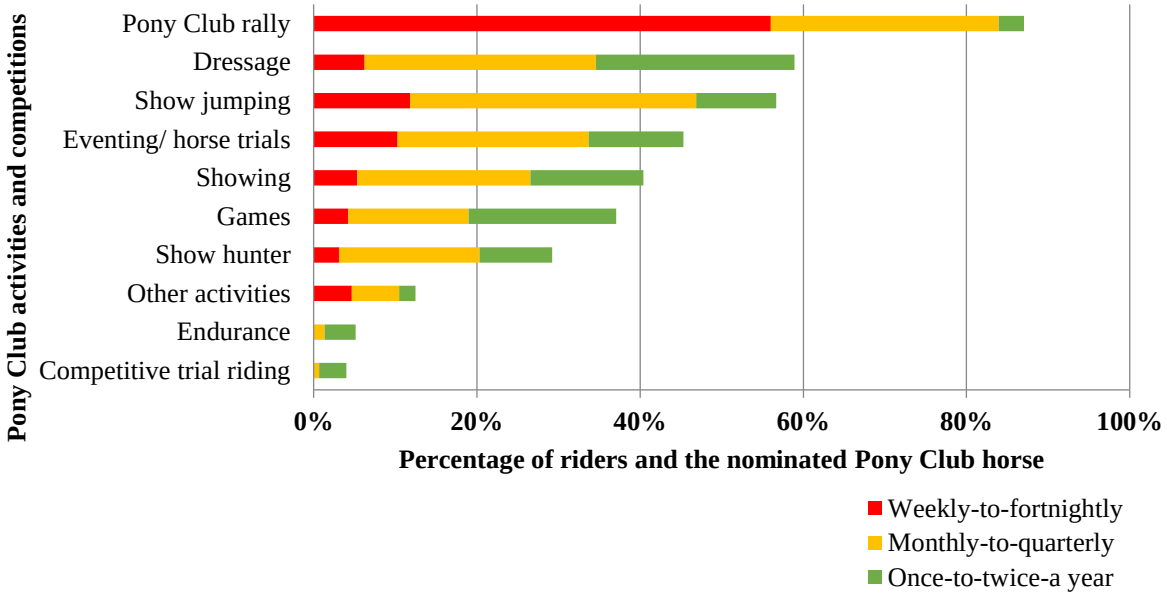


A-8. An excerpt from the Facebook page designed for the project, and used to broadcast the online survey to the Pony Club groups via Facebook.

A-9. An excerpt from Horsetalk: World equestrian news and information (horsetalk.co.nz) published on 24th December 2012, to encourage participation in the online Pony Club survey.

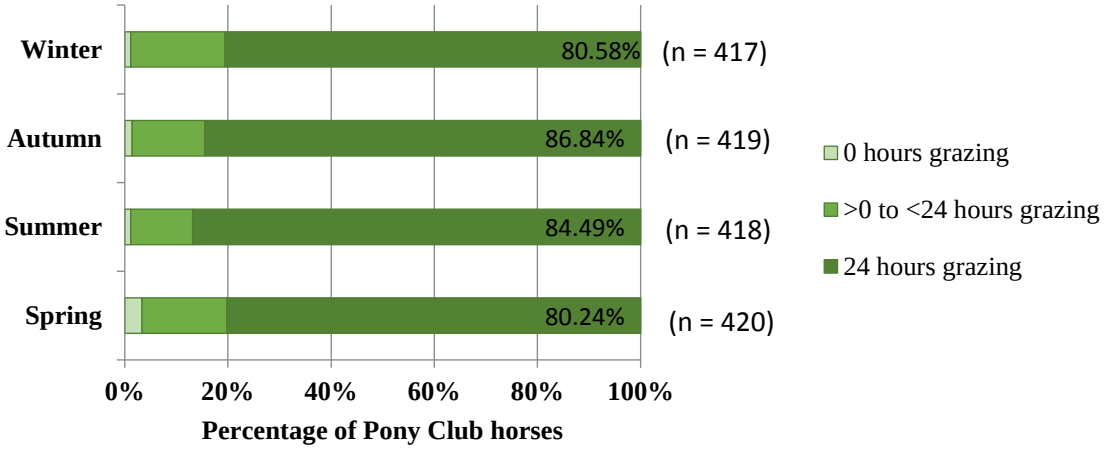
http://horsetalk.co.nz/2012/12/24/nz-pony-club-members-online-survey/#axzz3PXrLKa42

A-10. Participation of riders and the nominated Pony Club horse in various activities and competitions.



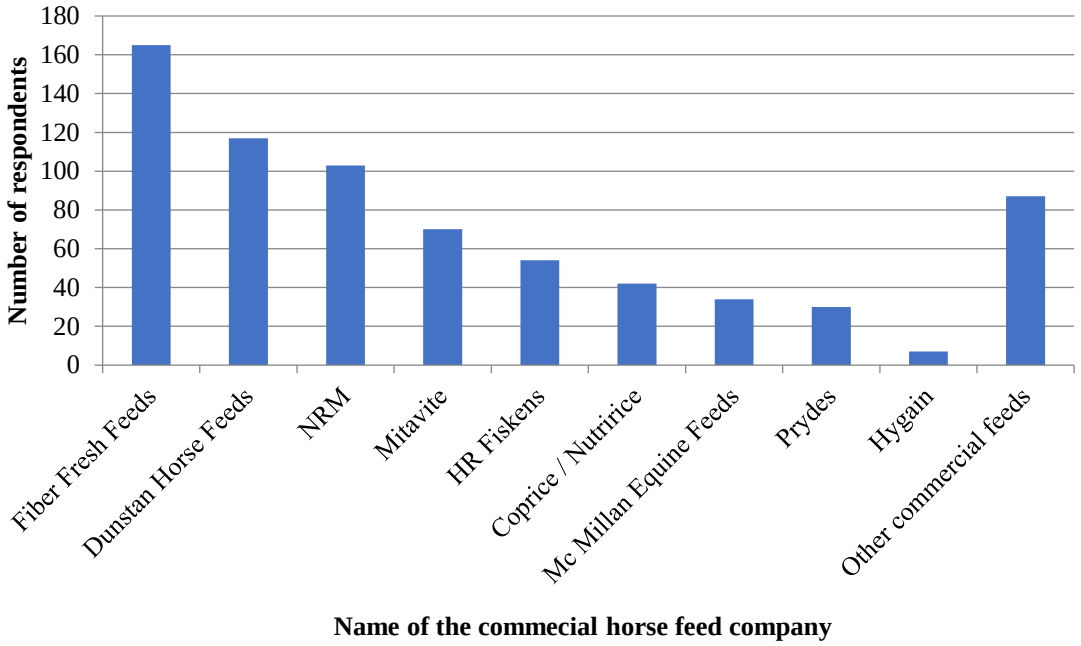
Legend: Riders participated in more than one activity or competition in the year. The remaining percentage of riders (to 100%) either did not participate in the activity or did not answer this question.

A-11. Seasonal grazing turnout for Pony Club horses



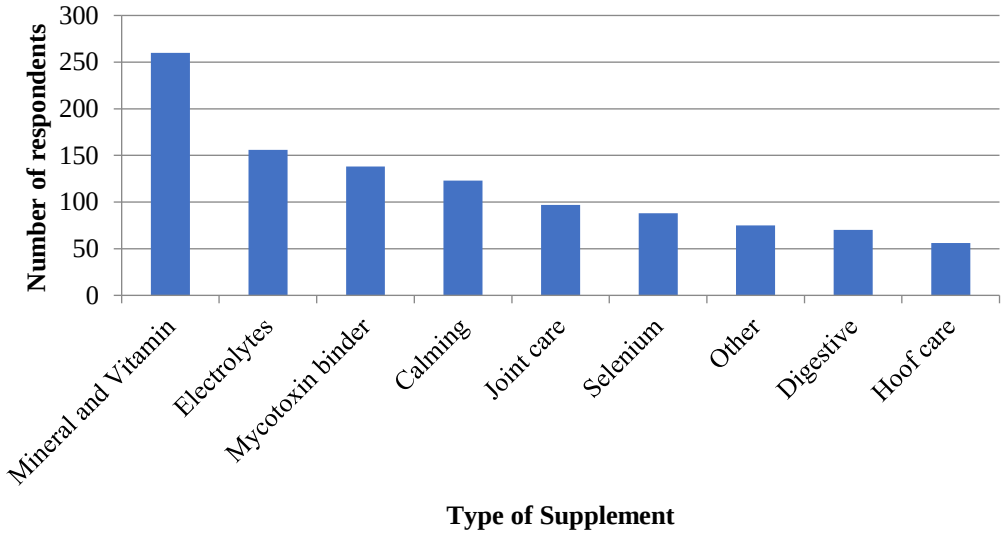
Legend: Most Pony Club horses were kept on pasture all year round, with little difference between the turnout periods for grazing across seasons.

A-12. Varieties of commercial horse feeds offered to Pony Club horses.



Legend: Most respondents offered their Pony Club horses a combination of commercially available horse feeds.

A-13. Supplements fed to Pony Club horses.



Legend: Most respondents fed a combination of supplements to their Pony Club horse.

A-14. Categorisation of commercially available premixed feeds offered to Pony Club horses and the digestible energy of the feed varieties

Commercially available premixed horse feeds					
High/ medium performance and growth*	**n**	**DE**	**Low performance, leisure and maintenance***	**n**	**DE**
dun_fiberplus	1	13.0	dun_coolfeed	41	11.8
dun_fibergrow	2	13.0	dun_all4feet	3	10.0
dun_grassbalancer	3	13.3	dun_spellingmix	1	13.5
dun_fiberbalancer	1	13.0	dun_maintenancemix	3	13.0
dun_eezymix	10	14.5	crt_coolfeed	25	11.0
dun_allyouneed	3	15.5	nrm_coolade	34	13.2
dun_sweetmix	1	14.5	nrm_horsenponypellets	21	12.6
dun_maximlowgi	4	13.5	nrm_litebrew	9	12.3
dun_coolfeedxtra	1	14.0	mvt_xtracool	14	11.0
dun_breedngrow	1	13.0	mvt_gumnuts	24	14.0
dun_brooodmaremix	2	13.5	dnh_safensound	1	8.5
crt_sporthorse	10	15.0	cop_coolconditioner	7	11.0
crt_freeup	1	16.0	cop_versatile	8	12.0
crt_rapidgain	4	16.0	cop_veteran	8	13.7
nrm_lowgisport	34	12.6	hrf_maintenancemix	33	13.0
nrm_sweetfeed	13	14.4	pry_easifeed150pellet	2	11.7
mvt_economixactive	20	12.0	pry_easiride	8	11.6
mvt_coolperformer	6	17.8	pry_easifeed4oldtimer	2	13.0
mvt_breeda	3	13.5	hyg_icecoolfeed	3	-
mvt_prosport	2	15.0	hyg_allrounder	2	-
cop_pperformancehorse	7	13.3	TOTAL	249	
cop_showncompetition	11	13.5	Number of varieties	20	
cop_staminanperformance	1	14.1			
hrf_horsebalancer	6	14.5			
hrf_broodmaremix	6	14.0			
hrf_yearlingmix	1	14.0			
pry_easisport	3	12.5			
pry_easiresponse	11	13.9			
pry_easiperformance	1	13.6			
pry_biomarecubes	2	14.2			
pry_easiresult	1	12.5			
hyg_zerolowgi	1	-			
TOTAL	173				
Number of varieties	32				

*Premixed feeds were categorised based on the recommendations of the feed manufacturer.

Legend: n - Number of respondents that offered the feed. DE - Digestible energy in MJ/kgDM (megajoules per kilogram dry matter), as reported by the feed manufacturer.

CHAPTER 4

BODY CONDITION AND MORPHOMETRIC MEASURES OF ADIPOSITY

(Face-to-Face Survey)

PRELUDE TO CHAPTER 4

Obesity is associated with metabolic disease and the risk of developing laminitis. Recent studies conducted on leisure horse and pony populations in many countries have highlighted the problem of equine obesity, with reported incidences of between 30-60%. The results of the preliminary online survey (Chapter 3) indicated that 22% of Pony Club horses and ponies in New Zealand were obese, and most of the animals were kept on pasture all year round. Given the seasonal changes in pasture growth and herbage mass availability, the potential for seasonal changes in body condition in this population of horses and ponies was great and required investigation.

Chapter 4 reports on the body condition and morphometric measures of adiposity of Pony Club horses and ponies in spring and autumn, and compared the subjective body condition scores rated by owners and the study personnel. The study collected data via a face-to-face survey of a cohort of horses and ponies ridden at Pony Club events in the lower North Island of New Zealand during spring and autumn (2013-14).

Supplementary information on the data recording sheets, the interview questionnaire and some additional findings are presented in the appendix for this chapter (Appendix B).

The key findings of the study were presented at the annual conference of the New Zealand Society of Animal Production (www.nzsap.org) in Dunedin on 1st July 2015, and the manuscript was published in the conference proceedings.

Fernandes, K.A., Rogers, C.W., Gee, E.K., Bolwell, C.F. and Thomas, D.G. (2015). Body condition and morphometric measures of adiposity in a cohort of Pony Club horses and ponies in New Zealand. *Proceedings of the New Zealand Society of Animal Production*, Dunedin, New Zealand. **75:** 195-199.

CHAPTER 4: BODY CONDITION AND MORPHOMETRIC MEASURES OF ADIPOSITY IN A COHORT OF PONY CLUB HORSES AND PONIES IN NEW ZEALAND

4.1 Abstract

A cross-sectional study was conducted to describe and compare the body condition and morphometrics of Pony Club (PC) horses and ponies at two points in the year. Data were collected from a cohort of PC riders in spring (n=158) and autumn (n=155), with 73 repeat measurements across the two seasons. There were more ponies (68%, 164/240, wither-height ≤148 cm) than horses in the study population. Most (76%, 183/240) animals were kept on *ad libitum* pasture throughout the year and were fed small quantities of supplementary feed. The median body condition score (BCS) of the animals was 6 (interquartile range 5-7). Forty-seven per cent (112/240) of the animals were scored 'fat' (BCS ≥7) and 25% (61/240) presented with a cresty-neck score ≥3. There was poor inter-rater agreement between the study personnel and owners for BCS of horses (Kappa=0.26) and ponies (Kappa=0.11). The study personnel identified 54% (88/162) of ponies as 'fat', whereas owners underestimated the BCS of ponies, and identified only 40% (65/162) as 'fat' (P<0.001). The measurements of body weight did not differ between seasons, and only some morphology-based assessments of adiposity differed between seasons. Overall, 17% (15/88) of 'fat' ponies and 21% (11/53) of those with 'cresty-necks' were reported to have had a previous history of laminitis. Laminar rings were observed in 98% (234/240) of the PC horses and ponies. The study highlighted that horses and ponies kept on pasture maintain body weight and condition from spring to autumn, but this finding may be biased by the high percentage of 'fat' ponies in the study population. The poor inter-rater agreement for BCS, particularly with ponies, and similar morphological measurements between seasons, highlights the challenges with using these measurements of adiposity in ponies.

4.2 Introduction

Leisure horses and ponies comprise the largest proportion of the New Zealand equine industry (Rosanowski *et al.*, 2012b). However, little information has been published on the management and health of animals in this sector. In contrast to the intensive management of race horses (Rogers *et al.*, 2007), preliminary data indicate that most sport and leisure horses and ponies in New Zealand are kept on pasture (Fernandes *et al.*, Verhaar *et al.*, 2014).

Ponies are efficient feed converters (easy-keepers), which enables them to maintain, if not gain, body condition when provided *ad libitum* pasture (Dugdale *et al.*, 2011). Recent studies have highlighted the growing problem of equine obesity, which has been reported in epidemic proportions in North America, United Kingdom and Australia with 30-60% of leisure horses and ponies affected (Buckley *et al.*, 2013, Robin *et al.*, 2013, Giles *et al.*, 2014). In New Zealand, a preliminary survey reported that 22% of Pony Club (PC) members scored their horse or pony with a body condition score (BCS) ≥7/9 (Fernandes *et al.*, 2015), but the true prevalence of equine obesity is unknown.

Assessing horse and pony body condition on a nine-point scale has been widely used to monitor body condition (Henneke *et al.*, 1983), and objective measurements such as girth:height ratio and neck circumference have been investigated for quantifying regional adiposity (Carter *et al.*, 2009). However, monitoring obesity remains challenging, perhaps due to the under-recognition of obesity by owners (Wyse *et al.*, 2008), or limitations in the current scoring and measurement systems (Carter *et al.*, 2009, Dugdale *et al.*, 2012).

Seasonal variations in body condition have previously been reported in feral and domestic horse populations (Scheibe and Streich, 2003, Giles *et al.*, 2014). However, it is unknown whether seasonal changes in body condition occur in leisure horses and ponies kept on pasture in New Zealand.

The objectives of the present study were to conduct a cross-sectional survey to obtain data on the feeding, management and morphometric measures of adiposity in PC horses and ponies, and to examine these measures for changes between spring and autumn.

4.3 Materials and Methods

Data were collected from PC riders attending events in the lower North Island of New Zealand during spring 2013 (November-December) and autumn 2014 (April-May) (Appendix B1 and B-2). The caretaker of the PC horse or pony provided data on the general management and health via a brief interview, after which their animal was examined to quantify weight, height, body condition, adiposity and hoof conformation (Appendix B-3 to B-5).

Body weight was obtained using a weigh platform (TruTest-703 electronic scales, Auckland, New Zealand; Appendix B-6), and height was measured with a spirit-level measuring stick (Equi-essentials, Kingston, MA, USA). Body condition score was estimated using a modified nine-point scale (Henneke *et al.*, 1983), and regional fat deposition along the neck (Cresty Neck Score, CNS) was assigned a score on a six-point scale (Carter *et al.*, 2009).

Morphometric measurements of the body and neck were recorded using a standard measuring tape (Easy-Measure, Taiwan; Appendix B-6), and included measurements of heart- and abdominal-girth circumference, body and neck length, neck circumference at 25%, 50% and 75% along the length of the neck, and neck-crest height (Carter *et al.*, 2009). All measurements were carried out by one author (KAF), and had coefficients of variation ≤1.5%. The hooves were examined for asymmetry between contralateral limbs and presence of laminar rings on the dorsal hoof walls (Hampson *et al.*, 2010).

Data were recorded on *pro-forma* recording sheets (Appendix B-4), and were entered into a database and screened for inconsistencies (MS Excel 2010, Microsoft Corporation, USA). Pony Club animals that measured ≤148 cm at wither-height were categorised as ponies, those >148 cm as horses, and animals with a BCS ≥7 were considered 'fat' (Carter *et al.*, 2009, Giles *et al.*, 2014).

Data were tested for normality using the Shapiro-Wilk normality test. Simple descriptive statistics are presented as mean ± standard deviation for parametric and median and interquartile range (IQR) for non-parametric data. Differences between seasons were tested using the paired t-test, or the Friedman's statistic for non-parametric variables. Associations with categorical data were tested with a Chi-squared test. Correlation between the owner-reported and author's measurements of height and weight were tested using Pearson's correlation coefficient (r). A Kappa test was used to compare the BCS rated by the

owner and the author. All statistical tests were analysed in STATA version 12.1 (STATA Corp, Texas, USA).

4.4 Results

Data were collected on 158 horses and ponies in spring and 155 in autumn (N=313, 73 repeats across seasons), from 10 Pony Clubs located within the lower North Island of New Zealand. The median age of these animals was 12 years (IQR 7-16), and 53% (126/240) were geldings. The animals had been owned for a median of one year (IQR 0.5-2.5) and were engaged in a variety of PC activities. The majority (68%, 164/240) of the animals were categorised as ponies, with a median height of 137 cm (IQR 122-146). A range of breed-descriptors were reported; the common pony breeds were Welsh or Welsh crosses (28%, 45/164) and mix-breed ponies (20%, 33/164), and over half the horses were Thoroughbred or Thoroughbred crosses (53%, 40/76).

Most horses and ponies (76%, 183/240) were kept on pasture throughout the year with 24-hour access to grazing, 25% of which (46/183) had restricted availability of pasture cover due to break-fencing or strip-grazing techniques (Table 4.1). The remaining animals spent a median of 12 hours (IQR 5-12) grazing and were yarded for parts of the day. Most (88%, 211/240) animals were fed some form of supplementary feed once daily. Hay and premixed feed were the most commonly reported supplementary feeds (Table 4.1).

The median BCS of the animals assessed by the author was 6 (IQR 5-7). Nearly half (47%, 112/240) of the animals were scored ‘fat’, and 25% (61/240) presented with a ‘cresty-neck’. Most of the animals identified as ‘fat’ or with a ‘cresty-neck’ were ponies (79%, 88/112 and 87%, 53/61, respectively).

Table 4.1. Feeding and management of Pony Club horses included in a study conducted during spring and autumn in the lower North Island of New Zealand.

Variables	%	n/N
Location		
Continuously on pasture 24 hours/day	76%	183/240
Unrestricted grazing	75%	137/183
Restricted grazing	25%	46/183
Partially yarded with restricted grazing-time	24%	57/240
Feeding supplementary feeds		
Type of supplementary feeds		
Grain	25%	53/211
Premixed feed	61%	128/211
Chaff	47%	99/211
Hay	68%	143/211
Other forages	25%	53/211
Feeding frequency		
Once daily	67%	141/211
Twice daily	18%	38/211
3-5 times a week	9%	19/211
Twice a week	6%	13/211
Estimated quantity per feeding (owner-reported)		
≤ 2 slabs of hay (~3 kg as fed)	82%	117/143
≤ 1 scoop of premixed feed (~1 kg as fed)	91%	116/128

There was a strong correlation (r=0.98) between the owner-reported height of the animals and that measured by the author, within both horses (r=0.91) and ponies (r=0.97) (P<0.001). The owner-estimated body weight correlated (r=0.85) with those measured by the author, with higher correlation coefficients within ponies (r=0.79) than horses (r=0.57) (P<0.001) (Appendix B-7 and B-8). The inter-rater agreement for the author- and owner-estimates of BCS was fair (Kappa=0.26) for horses and slight (Kappa=0.11) for ponies (Appendix B-9). There was a tendency for owners to underestimate the BCS of 'fat' horses and ponies, and overestimate the BCS for lean ponies with a bias towards a moderate BCS of 5/9.

When BCS was treated as a binary variable, a significantly higher percentage of ponies were scored 'fat' by the author (54%, 88/162) compared to the owners (40%, 65/162, P<0.001), a pattern which was also observed in horses (31%, 24/78 and 26%, 20/78 for author and owners, respectively, P<0.001). The inter-rater agreement between the author and owners for scoring an animal as 'fat' was moderate for horses (Kappa=0.43) and fair for ponies (Kappa=0.36).

For the subset of animals with repeated measures between seasons, there was no significant difference between the body weight in spring and autumn for horses (547±67 kg vs. 546±71 kg) ($P>0.05$), or ponies (353±105 kg vs. 350±102 kg) ($P>0.05$). There were some significant differences in the body and neck measurement values between spring and autumn (Table 4.2), but there was poor correlation for the morphometric measurements, particularly the abdominal-girth circumference, body length and neck-crest height, when compared between seasons.

Eighty-seven per cent of owners (209/240) were aware of the health history of their horse or pony. Only 10% (21/209) of owners reported that their animal was prone to laminitis, either due to an underlying condition or due to the occurrence of previous episodes of laminitis. Twenty (out of 21, 95%) of these laminitis-prone animals were ponies. Overall, 17% (15/88) of ponies that were 'fat', and 21% (11/53) of those with 'cresty necks' were reported to have had a previous history of laminitis.

Of the owners that reported at least one health issue with their animal in the previous year (49%, 103/209), lameness was most commonly reported due to musculoskeletal injury, hoof infections, laminitis or other known or unknown causes (Table 4.3). A veterinarian had diagnosed 9/14 of the reported cases of laminitis. Seven of the vet-diagnosed cases and all five owner-suspected cases of laminitis occurred in spring.

On examination of hoof conformation, 51% of the animals showed asymmetry between hooves of the contralateral limbs (Table 4.3). Ninety-eight per cent (234/240) of the horses and ponies showed the appearance of laminar rings on the dorsal surface, most of which had ≥ 3 rings on at least one hoof.

Table 4.2. Seasonal comparison of the author-recorded morphometric measurements of Pony Club horses included in a study conducted during spring and autumn in the lower North Island of New Zealand.

	Ponies					**Horses**				
	Spring		Autumn			Spring		Autumn		
	Median	IQR[1]	Median	IQR	P value	Median	IQR	Median	IQR	P value
Subjective scores by author										
BCS[2]	7	(5-8)	6	(5-7)	0.14	7	(5-7)	5	(5-6)	0.12
CNS[3]	2	(1-3)	2	(2-3)	**0.01**	1	(1-1)	1	(1-2)	**<0.001**
Objective measurements										
Body Measurements										
BMI[4]	0.019	(0.018-0.021)	0.018	(0.017-0.020)	**<0.001**	0.023	(0.022-0.024)	0.022	(0.020-0.023)	0.22
Girth:height	1.23	(1.20-1.26)	1.23	(1.21-1.25)	0.08	1.20	(1.19-1.23)	1.20	(1.17-1.22)	0.52
Abdominal girth:height	1.27	(1.23-1.33)	1.25	(1.21-1.33)	0.28	1.25	(1.20-1.30)	1.21	(1.16-1.28)	**<0.001**
Girth:length	1.24	(1.21-1.29)	1.20	(1.17-1.24)	**<0.001**	1.27	(1.21-1.30)	1.23	(1.15-1.28)	0.06
Abdominal girth:length	1.30	(1.24-1.35)	1.24	(1.20-1.28)	**0.002**	1.29	(1.24-1.36)	1.23	(1.18-1.29)	**0.02**
Neck measurements										
Neck crest height (cm)	10	(8-12)	14	(12-14)	**<0.001**	9	(9-10)	12	(11-14)	**<0.001**
Mean NC[5] (cm)	87	(80-92)	87	(78-92)	0.22	99	(95-100)	96	(90-99)	0.06
50NC[5] (cm)	85	(80-92)	84	(77-91)	**0.04**	99	(93-100)	94	(85-99)	0.17
Mean NC:height	0.008	(0.007-0.009)	0.008	(0.007-0.009)	0.64	0.007	(0.007-0.007)	0.007	(0.006-0.007)	0.07
50NC:height	0.64	(0.61-0.67)	0.63	(0.59-0.66)	0.07	0.61	(0.59-0.65)	0.60	(0.56-0.62)	0.17

[1]Interquartile range.
[2] Body condition score (1-9 scale).
[3] Cresty-neck score (0-5 scale).
[4] Body Mass Index calculated by body weight / (length x height).
[5] Neck circumference (NC), neck circumference at 0.5 of neck length (50NC).

Table 4.3. Health history and hoof characteristics of Pony Club horses included in a study conducted during spring and autumn in the lower North Island of New Zealand.

Variables	%	n/N
Health issues in the year preceding the study		
Absence of health issues	51%	106/209
Presence of health issues	49%	103/209
Lameness	71%	73/103
Musculoskeletal injuries	43%	31/73
Hoof infections	29%	21/73
Laminitis	19%	14/73
Vet-diagnosed	12%	9/73
Owner-suspected	7%	5/73
Other causes of lameness	11%	8/73
Gastrointestinal issues	10%	10/103
Colic	5%	5/103
Grass staggers	10%	10/103
Behavioural issues	9%	9/103
Hoof characteristics		
Shoeing		
Shod	44%	106/240
Bare hoof	56%	134/240
Hoof symmetry between contralateral limbs		
Symmetrical	51%	123/240
Asymmetrical	49%	117/240
Appearance of hoof rings		
1-2 rings	44%	102/234
≥ 3 rings	56%	132/234

4.5 Discussion

Data obtained on the feeding and management of PC horses and ponies in the present study were comparable to those obtained in a previous nationwide online survey (Fernandes *et al.*). Pony Club animals were kept on pasture all year round, which is similar to the management of Sport Horses (Verhaar *et al.*, 2014), emphasising the importance of pasture as a major dietary component for horses and ponies in New Zealand.

Horses and ponies in the present study showed limited changes in body weight and measures of adiposity between seasons, despite continuous access to pasture. In contrast, the percentage of obesity in a cohort of outdoor-living domestic horses and ponies was reported to be higher in summer than winter (Giles *et al.*, 2014). The difference in observations may be due to the high percentage of ‘fat’ ponies observed in our PC population, perhaps due to

the high proportion of ponies that were identified as Welsh, Welsh crosses and mix-breed ponies. 'Fat' ponies are often resistant to weight-loss when compared to horses (Argo *et al.*, 2012), and can maintain body weight even when the availability of pasture is limited (Dugdale *et al.*, 2011). Furthermore, the PC members may have regulated the quantity of pasture available, which perhaps facilitated the maintenance of body condition through spring and autumn. However, data on the digestible energy from pasture or supplementary feeds were not recorded in the study due to the difficulties with accurately quantifying feed intake under field conditions.

The poor agreement between the owner and author's estimates of BCS in the present study, may reflect the subjective nature of BCS scoring systems (Henneke *et al.*, 1983), perhaps due to the difficulties with differentiating scores >7/9 in obese ponies (Dugdale *et al.*, 2012), or because the owners provided a score based on an illustrated chart rather than comprehensive descriptions and palpation. Some owners did not recognise that their animal was 'fat', which may have been due to a tendency to score towards normalcy (BCS 5) or an owner's preference for over-conditioned ponies (Wyse *et al.*, 2008, Martin and Crowley, 2009).

Objective measurements of body and neck morphology could theoretically reduce the inaccuracies observed with subjective scoring (Carter *et al.*, 2009). While measurements of neck circumference seemed useful to compare neck-adiposity over time in our study, measurement of girth:height ratio appeared to be a poor indicator of obesity in ponies, in part because deposition of fat occurs predominantly on the rump rather than the girth region (Westervelt *et al.*, 1976). Monitoring adiposity in ponies remains challenging, perhaps due to under-recognition of obesity by the owners combined with the inherent physiology of weight-loss resistance and patterns of adiposity in ponies, and this merits further investigation.

The presence of laminar rings in our population could indicate the occurrence of low-grade inflammation, but their presence was not limited to the sub-group with a known history of laminitis. The appearance of laminar rings in feral horses has been associated with variations in dietary composition (Hampson *et al.*, 2010). Given the reported seasonal variations in pasture in New Zealand (Hirst, 2011), the appearance of laminar rings could indicate that horses and ponies react to changes in pasture, and this may also require further investigation.

4.6 Conclusion

The study highlighted that horses and ponies kept on pasture maintained body weight and condition from spring to autumn, but this finding may be biased by the high percentage of 'fat' ponies in the study population. The poor inter-rater agreement for BCS, particularly with ponies, and similar morphological measurements between seasons, highlighted the challenges with using these measurements of adiposity in ponies.

4.7 Acknowledgements

The authors thank the NZPCA members for their cooperation and participation in the study, and the students who assisted with data collection. The first author (KAF) was supported by a Massey University doctoral scholarship and the project was supported by a research grant from the Ministry of Business, Innovation and Employment in collaboration with Fiber Fresh Feeds Ltd. (NZ), for which we are grateful.

Appendix B

This appendix provides supplementary information on the survey design and data collection methods and some results presented in Chapter 4.

Survey design and data collection methods

B-1. Copy of the invitation and information sheet given to to Pony Club members.

B-2. Location of the Pony Clubs visited in the Lower North Island of New Zealand.

B-3. Copy of the survey questionnaire.

B-4. Copy of the data recording sheet.

B-5. Copy of body condition score and cresty-neck score charts.

B-6. Measuring body weigh on weight scales and morphometrics using a tape.

Additional Results

B-7. Scatter plot of body weight measured in spring versus autumn.

B-8. Scatter plot of owner-reported body weight versus body measured on electronic scales by the study personnel.

B-9. Distribution of the owner- versus author-estimated body condition scores for ponies.

B-1. Copy of the invitation and information sheet given to to Pony Club members.

WHAT TO DO?

PARTICIPATE

- Sign a form to confirm your voluntary participation in the study

INTERVIEW (5 Minutes)

- About your riding experience
- Feeding & exercising your pony
- Health & management of your pony

A CLOSER LOOK AT YOUR PONY

- Weigh & measure your pony
- Coat & hoof health
- Take a picture

Acknowledgements

THE NEW ZEALAND PONY CLUB NZPCA

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Ministry of Business, Innovation & Employment

Science + Innovation

INVITATION

To participate in a study on health and management of horses and ponies ridden at Pony Clubs in the Lower North Island of New Zealand

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Dear Pony Club riders and parents,

We are conducting a field study on the health and management of horses and ponies ridden at pony club events in the lower North Island of New Zealand.

You are invited to participate in a 5-minute interview comprised of questions relating to the health, feeding, exercise, and general management of your horse / pony.

We would also like to weigh and measure the height of your horse / pony. Please take this opportunity to get your horse/ pony weighed on a potable weighing scale at our interview.

Your participation is much appreciated!

Look forward to seeing you at the Rally

Kind regards,

Karlette

Contact:
Karlette Anne Fernandes
PhD Researcher
Email K.A.Fernandes@massey.ac.nz
Address Institute of Food, Nutrition and Human Health
Massey University, Private Bag 11222
Palmerston North 4442, New Zealand
Website www.massey.ac.nz

INFORMATION SHEET

The Equine Team at Massey University is passionate about improving the health of horses and ponies. With this in mind, we would like to explore further into specific health issues amongst recreational riding horses and ponies in New Zealand.

This study aims to collect information on the feeding, exercise, management, and health care of horses and ponies involved in pony club rallies in the lower North Island of New Zealand.

General Instructions:

1. All responses to the survey will be kept anonymous; any personal information will remain strictly confidential.

2. Pony club riders must be 16 years of age or older to participate in the interview.

3. Riders under the age of 16 years will require a parent or guardian to participate in the interview on their behalf.

4. The interview will only take 5 minutes, followed by a physical assessment (measurements) of your horse / pony.

5. Please contact the researcher for any queries in person, or via the e-mail address provided.

A summary of results will be available via the New Zealand Pony Clubs Association newsletter.

Project contact details:

PhD Researcher (Student):
Karlette A. Fernandes K.A.Fernandes@massey.ac.nz

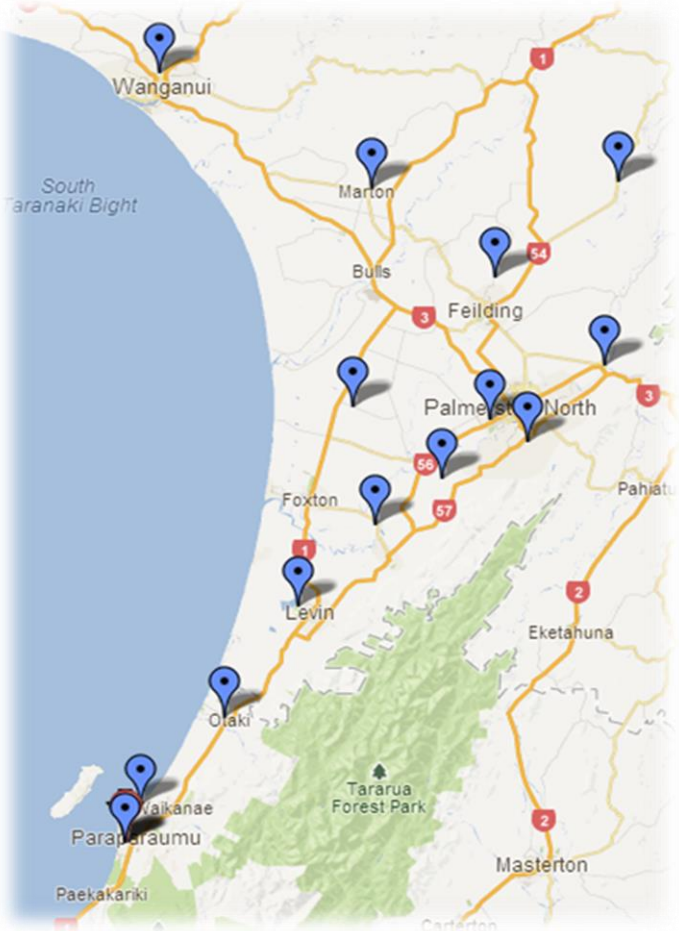
Supervisors:
Dr. David G. Thomas D.G.Thomas@massey.ac.nz
Dr. Erica K. Gee E.K.Gee@massey.ac.nz
Dr. Chris W. Rogers C.W.Rogers@massey.ac.nz

Human Ethics Statement

This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researchers named below are responsible for the ethical conduct of this research. If you have any concerns about the conduct of this research that you wish to raise with someone other than the researchers, please contact:

Professor John O'Neill
Director, Research Ethics
Telephone +64 06 350 5249
Email: humanethics@massey.ac.nz

B-2. Location of the Pony Clubs visited in the Lower North Island of New Zealand.



Wanganui-Petre Pony Club

Nga Tawa Equestrian Academy

Ashhurst-Pohangina Pony Club

Manawatu Pony Club (Kimbolton, Feilding, Opiki, Tiritea, Palmerston North)

Stanway Pony Club

Tekawau Pony Club

Moutoa Pony Club

Levin Pony Club

Otaki Pony Club

Kapiti Pony Club

B-3. Copy of the survey questionnaire.

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NZPCA eHAM Field Study

Rider & Pony Name: _____
Pony Club: _____ Date: _____

RIDER'S DETAILS

Rider's name: _____ **Gender**: Male / Female

Age: _____ years

ANIMAL'S DETAILS

Horse/ Pony's Name: _____ **Gender**: Mare / Gelding

Age: _____ years

Breed: _____

Questions about the Rider & Pony

Q: What level of riding certificate do you currently hold at Pony Club?

(Please circle one) None D D+ C C+ B A Riding A H

Q: How long have you been riding horses / ponies? _____ years

Q: How long have you owned this horse/ pony? _____ years

Q: What is its height? _____(cm or hands)

Q: How much does it weigh? _____ kg

Q: In the last week, where was your pony kept, and for how long?

☐ Paddock _____ ☐ Yard _____ ☐ Stable _____

Q: Was your pony clipped at the start of this season? _____ (Yes / No)

Q: If clipped, what type of clipping? *(Please tick one)*

☐ Full-clip ☐ Hunter ☐ Blanket ☐ Chaser ☐ Trace ☐ Strip ☐ Don't know

Q: On the scale of 1-9 how would you body condition score your pony?

(Please circle one number; if unsure, ask the interviewer for a chart)

1 2 3 4 5 6 7 8 9

Q: Would you be interested in participating in future nutrition studies relating to your pony's health? ☐ Yes ☐ No

Contact details:
NAME (Rider / parent): _____

e-mail: _____ Tel. Ph: _____

INTERVIEW NUMBER: _____

B-4. Copy of the data recording sheet.

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NZPCA eHAM Field Study – INTERVIEW

Rider & Pony Name: _____
Pony Club: _____ Date: _____

Work / exercise in the last week:

Type - hacking (HK), flatwork (FL), lunging (LN), jumping (SJ), games or competitions (CT)

Activity / frequency	Mon	Tue	Wed	Thurs	Fri	Sat	Sun
Type **Amount of time** (in min / hrs):							

Feeding in last week:

Feed type	Quantity / scoop size	Frequency per day
Pasture		
Grain		
Hay		
Haylage / baleage		
Supplements		

Health problems in last year:

Disorder	Time + Frequency	Reason
Lameness *(such as laminitis)*		
Digestive upset *(such as colic)*		
Grass Staggers		
Behavioural issues *(such as weaving, crib-biting, wind sucking)*		

Coat appearance in last year: _____

_____

☐ Smooth/shiny ☐ Dull/rough ☐ Cover Yes / No

Did it have a long-curly coat in summer? Yes / No

Comments: Did the drought affect you? What feeding strategy did you adopt?

Karlette Anne Fernandes

INTERVIEW NUMBER: _____

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NZPCA eHAM Field Study

Rider & Pony Name: _____
Pony Club: _____ Date: _____

PHYSICAL EXAMINATION

Measurements:

Weight: _____ kg BC Score: _____ CN Score: _____

Neck-Length: _____ cm Neck Crest-height @.50 NL: _____ cm

Neck-circumference: @.25 NL _____ cm

@.50 NL _____ cm

@.75 NL _____ cm

Body Length: _____ cm Height: _____ cm

Heart-girth: _____ cm Abdominal-girth: _____ cm

Fat deposits: ☐ Orbital ☐ Neck ☐ Withers ☐ Shoulders ☐ Ribs

☐ Rump ☐ Loin ☐ Tail-head ☐ Ventral abdomen

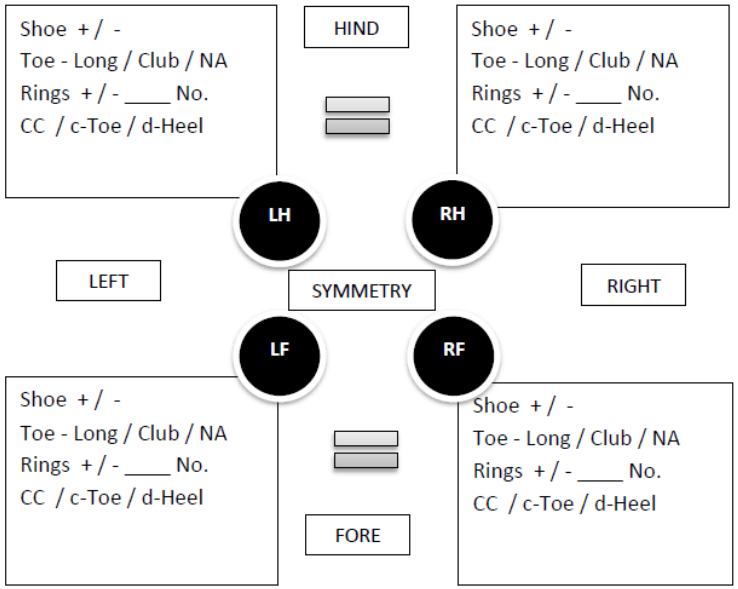
Coat appearance:

☐ Smooth / Rough ☐ Shiny / Dull ☐ Clipped / full-coat

Hirsutism: ☐ Absent ☐ Generalised ☐ Regional _____

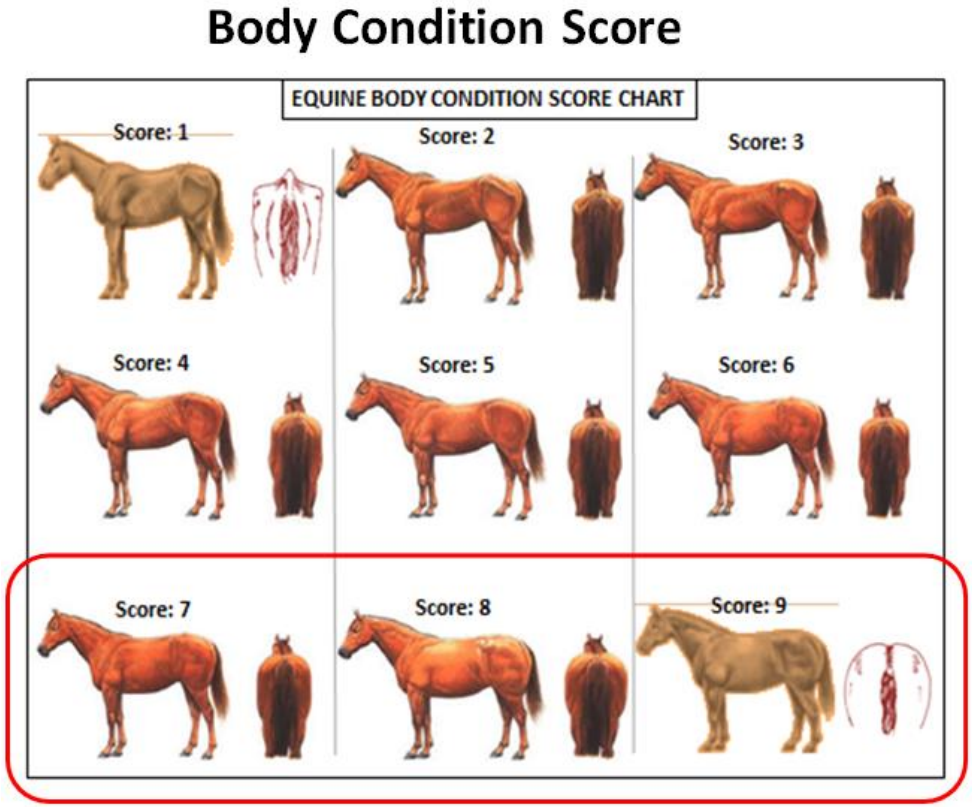
Pelage fibre length (mm): Mid-neck _____ Mid-ribs _____

Hoof Conformation:

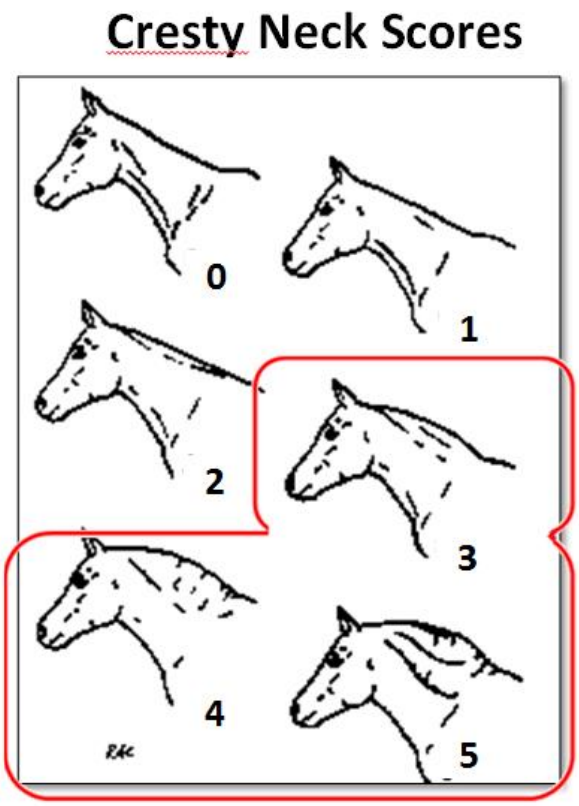


Comments: _____

B-5. Copy of body condition score and cresty-neck score charts.



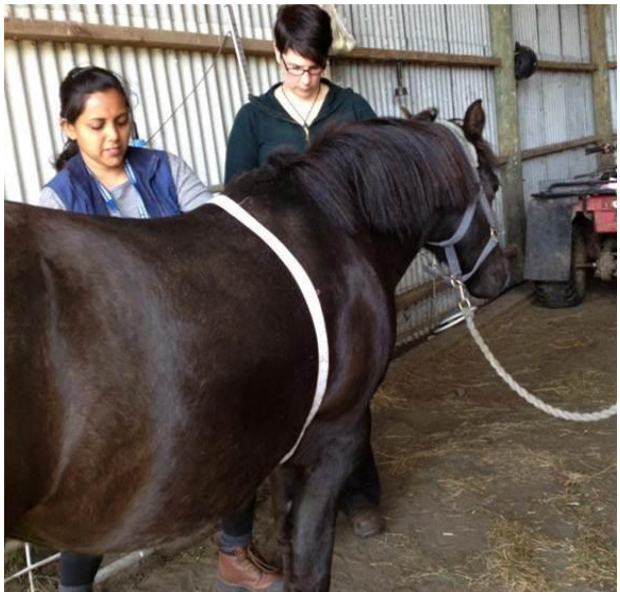
BCS ≥7 "fat"



CNS ≥3 "cresty neck"

B-6. Measuring body weight on electronic weigh scales and morphometric measurements using a tape (cm).

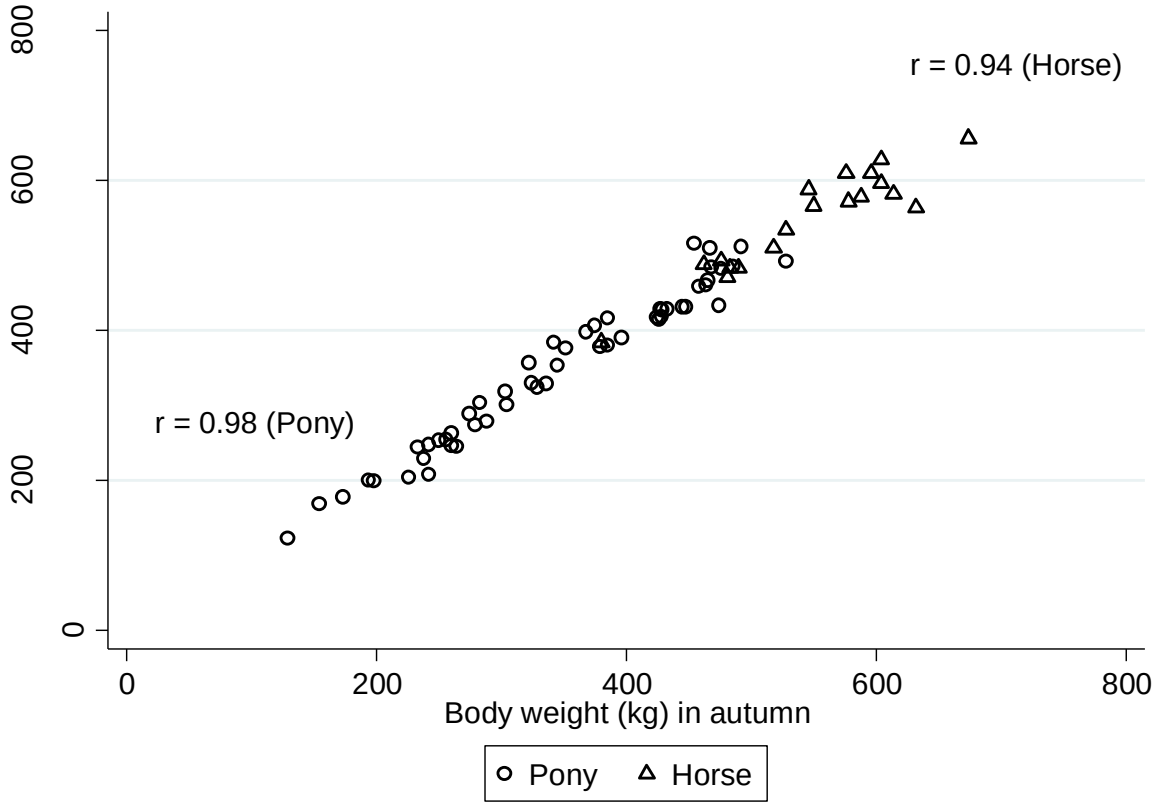
(A) Measuring weight (kg)



(B) Measuring body circumference (cm)

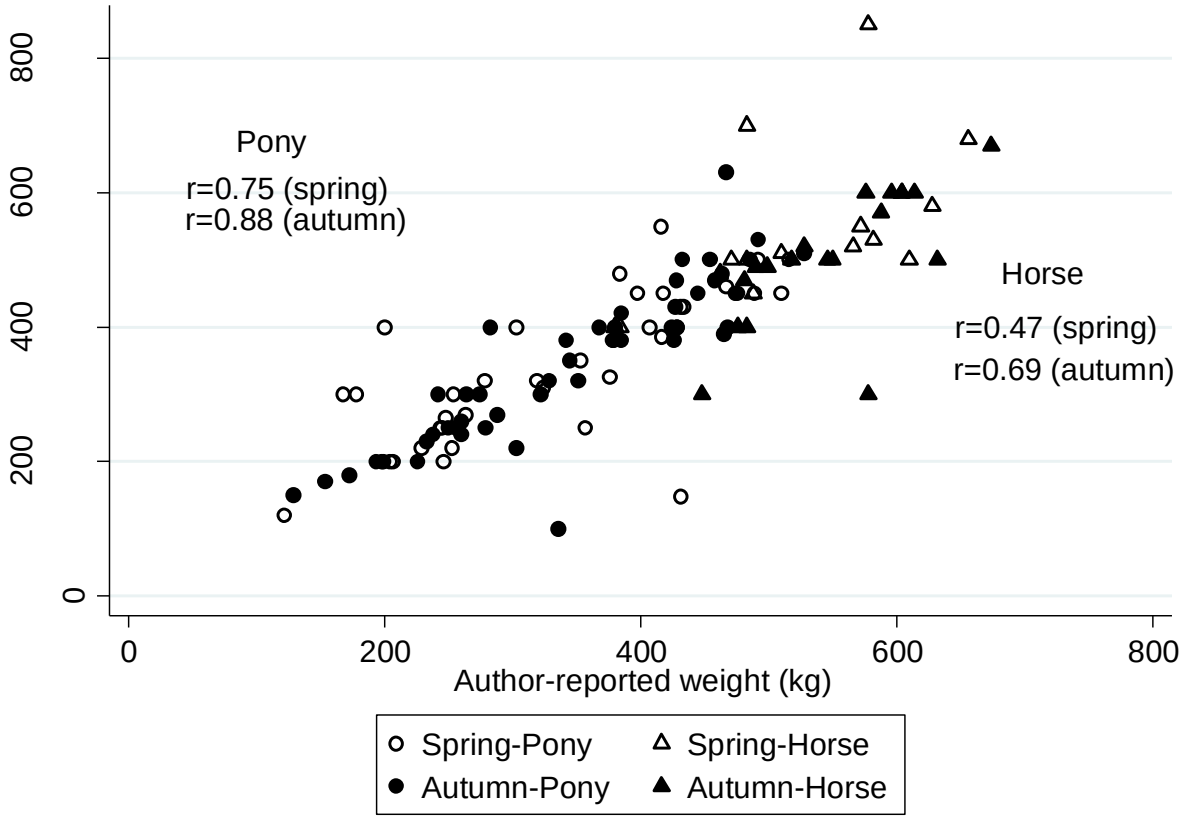
(C) Measuring crest neck height (cm)

B-7. Scatter plot of body weight measured in spring versus autumn.



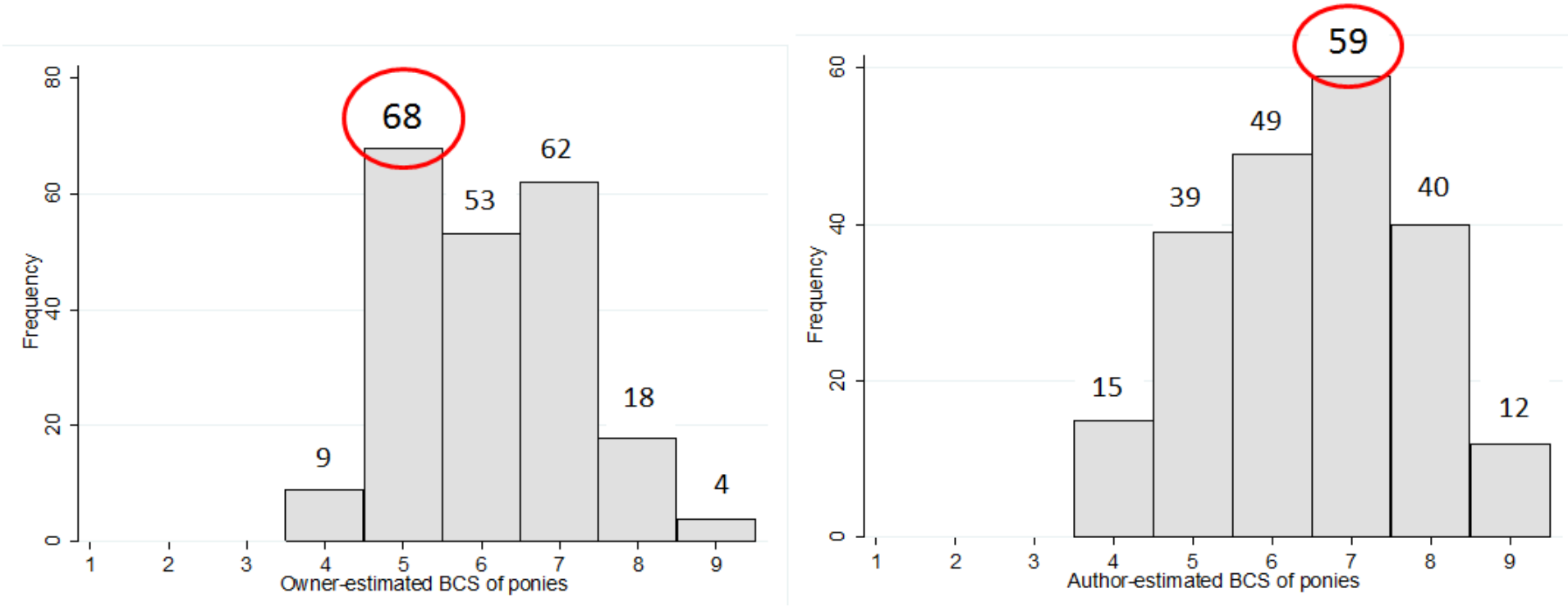
Legend: There was strong correlation between the body weight measuring in spring versus autumn, for both horses and ponies, indicating limited change in body weight of the horses and ponies from spring to autumn.

B-8. Scatter plot of owner-reported body weight versus body weight measured on electronic scales by the study personnel.



Legend: There was stronger correlation between the owner-estimated and author-reported body weight of ponies when compared to horses.

B-9. Distribution of the owner- versus author-estimated body condition scores for ponies.



Legend: In comparison to the author-estimated body condition scores, owners had the tendency to under-estimate the body condition score of their ponies.

CHAPTER 5

FAECAL MICROBIOTA OF HORSES FOLLOWING DIETARY TRANSITION

(Yearling Horse Trial)

PRELUDE TO CHAPTER 5

The results of the two Pony Club surveys (Chapters 3 and 4) indicated that most horses in New Zealand were kept on pasture all year round, with unrestricted access to grazing and supplementary feeding of a variety of commercial feeds. Many of these leisure horses and ponies showed signs of obesity and almost all had rings on their hooves. Given the seasonal changes in pasture growth and composition, and the abrupt dietary changes with the incorporation of supplementary feeds, it was hypothesised that these factors may influence the population of faecal microbiota in horses and this required investigation.

The experiment in Chapter 5 utilised a sensitive sequencing technique (454 Titanium Pyrosequencing) to examine the changes occurring in the microbial populations that were obtained from genomic DNA extracted from the faecal samples collected during the study. Chapter 5 reports on the composition of the microbiota of pasture-fed horses in New Zealand, and compared the microbiota profiles following abrupt dietary change. Both these aspects have not been previously investigated.

Supplementary information on the methodology and results of data analysis are presented in the appendix for this chapter (Appendix C). Chapter 5 is based on a manuscript published in the Journal Public Library of Science One (PLoS ONE).

Fernandes, K.A., Kittelmann, S., Rogers, C.W., Gee, E.K., Bolwell, C.F., Bermingham, E.N. and Thomas, D.G. (2014). Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. *PLoS ONE* **9**(11): e112846.

CHAPTER 5: FAECAL MICROBIOTA OF FORAGE-FED HORSES IN NEW ZEALAND AND THE POPULATION DYNAMICS OF MICROBIAL COMMUNITIES FOLLOWING DIETARY CHANGE

5.1 Abstract

The effects of abrupt dietary transition on the faecal microbiota of forage-fed horses over a 3-week period were investigated. Yearling Thoroughbred fillies reared as a cohort were exclusively fed on either an ensiled conserved forage-grain diet ("Group A"; n = 6) or pasture ("Group B"; n = 6) for three weeks prior to the study. After the Day 0 faecal samples were collected, horses of Group A were abruptly transitioned to pasture. Both groups continued to graze similar pasture for three weeks, with faecal samples collected at 4-day intervals. DNA was isolated from the faeces and microbial 16S and 18S rRNA gene amplicons were generated and analysed by pyrosequencing. The faecal bacterial communities of both groups of horses were highly diverse (Simpson's index of diversity > 0.8), with differences between the two groups on Day 0 ($P < 0.017$ adjusted for multiple comparisons). There were differences between Groups A and B in the relative abundances of four genera, BF311 (family Bacteroidaceae; $P = 0.003$), CF231 (family Paraprevotellaceae; $P = 0.004$), and currently unclassified members within the order Clostridiales ($P = 0.003$) and within the family Lachnospiraceae ($P = 0.006$). The bacterial community of Group A horses became similar to Group B within four days of feeding on pasture, whereas the structure of the archaeal community remained constant pre- and post-dietary change. The community structure of the faecal microbiota (bacteria, archaea and ciliate protozoa) of pasture-fed horses were also identified. The initial differences observed appeared to be linked to recent dietary history, with the bacterial community of the forage-fed horses responding rapidly to abrupt dietary change.

5.2 Introduction

The horse is a cursorial grazer with the ability to efficiently utilise high-fibre grass and other forages (Janis, 1976, Ley *et al.*, 2008). The majority of ingested plant fibre is comprised of structural carbohydrates such as cellulose, hemicellulose, and lignin, which cannot be digested by host enzymes in the foregut. As a result, the undigested plant material reaches the hindgut where breakdown of cellulose and hemicellulose occurs through the process of microbial fermentation, generating energy-yielding products such as volatile fatty acids (VFAs) (Cymbaluk, 1990, Vermorel and Martin-Rosset, 1997). It is estimated that forage-fed horses may obtain 50-70% of their energy requirements from VFAs (Glinsky *et al.*, 1976).

Several species of microbes including bacteria, archaea and eukarya (protozoa and fungi) inhabit the equine gastrointestinal tract (Moore and Dehority, 1993, Al Jassim and Andrews, 2009). However, the bacterial community, which represents the major proportion of the hindgut microbiota, has been the focus of much of the published literature and has predominantly been investigated using culture-based techniques (Julliand *et al.*, 2001, Müller *et al.*, 2008, Costa and Weese, 2012). Other molecular techniques independent of culture, such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), fluorescent in-situ hybridization (FISH) and terminal restriction fragment length polymorphism (TRFLP), have also been used (Yamano *et al.*, 2008, Willing *et al.*, 2009, Costa and Weese, 2012). More recently, a few investigations have used metagenomics involving high-throughput next generation sequencing (pyrosequencing of 16S and 18S rRNA gene amplicons) to explore the equine hindgut microbiome (eHGM) *in vivo* (Costa *et al.*, 2012, Steelman *et al.*, 2012, Dougal *et al.*, 2013). However, there are still gaps in the current knowledge on the composition and the function of the microbial communities that inhabit the equine hindgut. This may be due to the difficulty in culturing anaerobic microorganisms and comparing results from studies that have used different experimental designs, sequencing methodologies and phylogenetic and statistical data analyses (Costa and Weese, 2012).

Because of the need to fistulate the horse to obtain hindgut samples, the majority of the work on the eHGM has focused on the use of material obtained from faecal samples (Costa and Weese, 2012). From the limited comparative work conducted on the bacterial community

of the equine hindgut and faeces, there appears to be good agreement between the microbiomes of the colon and faeces (Dougal *et al.*, 2012, Dougal *et al.*, 2013).

The population of hindgut microbiota is sensitive to changes occurring in the gastrointestinal environment, with variations in the bacterial community structure reported in response to the composition of a diet, passage rate of digesta, and level of pre-caecal starch digestion (Julliand *et al.*, 2006, Van Weyenberg *et al.*, 2006). Abrupt dietary changes, particularly the availability of fermentable starch and water-soluble carbohydrates, can perturb the populations of microbes in the hindgut and can lead to digestive and metabolic disorders such as hindgut acidosis, colic and laminitis (Elliott and Bailey, 2006, Milinovich *et al.*, 2006, Al Jassim and Andrews, 2009). Forage-only diets promote greater microbial stability, evident by the lower microbial counts and relative abundances of specific lactic acid producing bacteria (Willing *et al.*, 2009). However, the effects of changes in forage-based diets on the bacterial population in the hindgut or faeces may be dependent on the type of horse (breed), composition of the diet and management practices (Yamano *et al.*, 2008, Muhonen *et al.*, 2010, O' Donnell *et al.*, 2013).

Culture-based techniques have identified that faecal colony forming units of *Streptococcus* spp. and *Lactobacillus* spp. increase significantly from pasture baseline values within six days of an acute dietary transition to a grain-based diet, with numbers subsequently decreasing when the horses were returned back to grazing on pasture (van den Berg *et al.*, 2013). Similarly, an abrupt change from feeding 100% hay diets to a combination of hay and concentrates was reported to produce changes in the hindgut bacterial populations within five hours (caecum) and 29 hours (colon), with greater changes occurring in the colon (de Fombelle *et al.*, 2001). More recently, an *in vitro* study suggested that bacterial communities isolated from horse faeces responded to a carbohydrate substrate within 12 hours, with significant changes in relative abundances of bacteria over a 48-hour period (Biddle *et al.*, 2013). The above findings indicate that changes in the bacterial community occur rapidly within the hindgut, and may be evident in the faeces within a few days, depending on the type of diet and the transit time through the intestinal tract.

In New Zealand, equine pastures are predominantly comprised of a perennial ryegrass and white clover mix, with lesser quantities of other grasses and legumes, and many horses are kept or reared on pasture all year round (Hoskin and Gee, 2004, Rogers *et al.*, 2007, Hirst, 2011). The composition of microbiota in the faeces of New Zealand pasture-fed horses, or the

effects of an abrupt dietary change from forage-grain diets to pasture on the hindgut or faecal microbiota, are currently unknown. The aims of the current study were to: 1) describe and compare the faecal microbiota of horses grazing on pasture to those fed exclusively on an ensiled conserved forage-grain diet in loose-boxes; and 2) investigate the changes in the relative abundance of faecal microbiota due to abrupt dietary change from an ensiled conserved forage-grain diet to pasture over a period of three weeks.

5.3 Materials and Methods

5.3.1 Ethics statement

The use of animals, including welfare, husbandry, experimental procedures, and collection of the faecal samples for this study, was approved by the Massey University Animal Ethics Committee (MUAEC), Massey University, Palmerston North, New Zealand (Protocol number 12-51).

5.3.2 Experimental design and sample collection

Twelve yearling Thoroughbred fillies (mean age ± standard deviation [SD], 396 ± 22 days) born and reared as a cohort on a commercial Thoroughbred stud farm (Palmerston North, Manawatu, New Zealand) were enrolled in the study during the spring of 2012 (November). Ten of the yearlings were sired by the same stallion. The general health and history of feeding management were recorded, and included daily observations by the stud master for any signs of illness or disease, weekly measurements of height, weight and body condition scores, anthelminthic treatments, and veterinary check-ups. The yearlings were in good health and had a median body condition score of six (Interquartile range [IQR] 5-6) on a 9-point scale (Henneke *et al.*, 1983), and a mean height and weight of 146.3 ± 2.8 cm and 348.3 ± 21.7 kg, respectively.

The 12 yearling horses were randomly divided into two treatment groups. For 21 days prior to Day 0, horses in Group A (n=6) were kept in loose-boxes (4 × 4 m) lined with rubber matting, and were fed exclusively on a commercial ensiled conserved forage-grain-based ration (Diet F - FiberSure®, Fiber Fresh Feeds Ltd., Reporoa, New Zealand), as part of a voluntary feed intake and digestibility study [30] (Appendix C-1). Diet F comprised of ensiled chopped lucerne (*Medicago sativa*; alfalfa grass; 65%), cracked maize (*Zea mays*) grain (35%)

and a vitamin and mineral premix with molasses (5%); the nutrient analysis of the diet is given in Table S1 (Appendix C-2). The loose-boxes were arranged in a single row, the lower half of the internal walls were made of wood and the upper half made of wire mesh, allowing visual contact between all horses in the loose-boxes and the adjacent yard. The horses were turned-out for exercise in pairs, in a compact-earth yard adjacent to the loose-boxes, for 30 minutes twice a day (Bishop, 2013). Horses in Group B were kept in a paddock and were provided *ad libitum* pasture (Diet P; a standard New Zealand ryegrass-clover pasture comprised of ~80-95% perennial ryegrass (*Lolium perenne*) and ~5-20% white clover (*Trifolium repens*) (Hoskin and Gee, 2004)) for 21 days prior to Day 0 (Appendix C-1). After the Day 0 faecal samples were collected, horses in Group A were abruptly transitioned to feeding on pasture (Diet P). Both groups of horses continued to graze on pasture for the next three weeks (21 days), during which the horses were kept in separate 1.5-2.0 hectare paddocks on the same property, containing pasture of similar herbage mass (pasture cover of 1600-2000 kg DM /ha/year) and nutrient content (Appendix C-2, Table S1).

Faecal samples were collected from all yearlings between 0900 and 1200 hours on Day 0, and subsequently at 4-day intervals over a period of 21 days (giving a total of 72 samples). The samples were collected within two minutes of defecation, using a forceps to collect representative faecal samples with minimal environmental contamination. These were immediately transferred into 3 ml polyethylene cryovials (Ray Lab Ltd., Auckland, New Zealand), and snap frozen in liquid nitrogen. The faecal samples were stored in a portable canister containing liquid nitrogen and transferred to a -80 °C freezer within four hours of collection and stored until laboratory analysis.

5.3.3 DNA extraction, PCR amplification of target genes and pyrosequencing

Nucleic acids were extracted from 100 mg of faeces by disrupting the cells by a combined bead-beating and phenol-chloroform-isoamyl alcohol (25:24:1; vol:vol:vol) treatment and subsequent precipitation of proteins with chloroform (Lueders *et al.*, 2004). DNA was precipitated from the aqueous phase with two volumes of 30% (wt:vol) polyethylene glycol, washed with 70% (vol:vol) ice-cold ethanol, dried and eluted in 50 µl of elution buffer (EB; 10 mM Tris, pH 8.5 with HCl). Extracted DNA was quantified using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and normalized to 40 ng/µl.

Polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA genes (V1-V3 regions), archaeal 16S rRNA genes (V6-V8 regions), and ciliate protozoal 18S rRNA genes (V5-V8 regions) were carried out as described previously, using universal primers (Ba515Rmod1, Ar915aF and Reg1302R) for the three groups of microorganisms (Kittelmann *et al.*, 2013). All primers contained the 454 Life Science (Branford, CT, USA) adaptors A (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3') or B (5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3') for Titanium sequencing, and a unique 12-base error-correcting barcode was attached to adaptor A for sample identification (Fierer *et al.*, 2008, Caporaso *et al.*, 2011). A PCR master mix of 76 µl was prepared for each DNA sample (per microbial group), as previously described (Kittelmann *et al.*, 2013). An aliquot of 19 µl was transferred to serve as a no-template negative control. The remaining 57 µl of reaction mix were spiked with 10-40 ng of DNA contained in 3 µl of water, and then divided into three aliquots of 20 µl each. Amplification was performed in a Mastercycler proS (Eppendorf, Hamburg, Germany) using a previously described protocol (Kittelmann *et al.*, 2013). Triplicate PCR products were pooled, and correct sizes of PCR products and signal absence from the negative controls were verified by agarose gel electrophoresis.

Following quantification of PCR products using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and a fluorometer (BioTek Instruments, Winooski, VT, USA), amplicons of the same target gene and region were pooled into three separate pools and loaded onto a 1%-agarose gel (wt:vol) prepared with 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8 with NaOH). Bands were visualised under blue light transillumination, excised, and DNA purified from the gel slices with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Gel-purified amplicon pools were quantified in triplicate with the Quant-iT dsDNA HS assay kit (Invitrogen). The three amplicon pools were normalized to contain 1×10^9 copies μl^{-1} and subsequently mixed at a ratio of 5:1:1 (Bacteria, Archaea, Protozoa) (Kittelmann *et al.*, 2013). This final pool was sent to MWG Eurofins (Ebersberg, Germany) for Titanium pyrosequencing on a 454 Life Sciences Genome Sequencer FLX machine (454 Life Sciences, Branford, CT, USA).

5.3.4 Phylogenetic analysis of pyrosequencing reads

Samples were processed and analysed using the software QIIME (Quantitative Insights Into Microbial Ecology) v1.5 (Caporaso *et al.*, 2010b). Sequence reads were assigned to corresponding samples by examining the 12-nucleotide error-correcting Golay barcodes

using the *split_libraries.py* script in QIIME. The *split_libraries.py* script (with default settings) selected sequences that had a minimum average quality score of 25, a maximum of six ambiguous bases with no allowance for mismatches in primer sequence, a maximum homopolymer run length of six and a maximum sequence length of 1000 bp. All sequences that did not meet the quality-filtering criteria were excluded from downstream analysis. For bacteria and archaea, only sequences that were ≥ 400 bp in length (including primers, option -l), and in which both the forward and reverse primers were detected, were selected for further analyses. The primers were subsequently removed (option -z truncate_remove), and this option allowed only high-quality sequences to be retained, in which the reverse primer sequence was unambiguously detected. The remaining sequences that did not meet the quality criteria were removed from the bacterial and archaeal sequence libraries. Bacterial and archaeal 16S rRNA gene sequence data were denoised using Acacia (Bragg *et al.*, 2012) and chimera checked using the blast fragment method in QIIME (Altschul *et al.*, 1990, Caporaso *et al.*, 2010b) against the Greengenes database (gg_13_5/gg_13_5.fasta (McDonald *et al.*, 2011)). All sequences that did not meet the quality criteria, and those identified as pyrosequencing noise or potential chimeras, were removed from the bacterial and archaeal libraries used for further downstream analysis. Sequences stemming from ciliate 18S rRNA genes that were > 200 bp in length were truncated to variable lengths so that the average quality score was > 25 (all other parameters were set on the default option). The remaining sequences that did not meet the quality criteria were removed from the ciliate protozoal library.

Clustering of operational taxonomic units (OTUs) was performed using the uclust method (Edgar, 2010) for bacteria and archaea at a 97% similarity threshold, or the prefix-suffix method passing the option "-p 1000" for protozoa (QIIME team, unpublished). Representative OTUs were assigned to taxonomic ranks as follows: bacterial 16S rRNA genes were BLAST-searched against the Greengenes database (gg_13_5/gg_13_5.fasta (McDonald *et al.*, 2011)); archaeal 16S rRNA genes and protozoal 18S rRNA genes were BLAST-searched against a rumen specific, in-house database (Janssen and Kirs, 2008) and the Silva eukaryotes database v.111 (Quast *et al.*, 2013), respectively.

Of the samples collected in the study, 71/72 samples had at least 1000 bacterial sequences per sample. The remaining one sample had 268 sequences and was removed from the bacterial library used for further downstream data analysis. Within the archaeal library, 70/72 samples had at least 320 sequences per sample. The remaining two samples, which had 15 and 236 sequences, were removed from the archaeal library used for downstream data

analysis. Since PCR amplicons for ciliate 18S rRNA genes were obtained from only 36/72 DNA samples, we used a minimum sequence read cut-off of 250 sequences per sample to report ciliate diversity in 26/36 samples. Subsequently, the OTU tables were rarefied at 1,000 (bacteria), 320 (archaea) and 250 (ciliate protozoa) sequences per sample and relative abundance tables were obtained at the phylum-, family- and genus-levels (bacteria), a mixed-taxon-level (archaea), or genus-level (ciliate protozoa).

To access the richness of microbial species captured within the samples, collector's curves for bacteria, archaea and ciliate protozoa communities were constructed from the OTU tables generated in QIIME, by using the *alpha_diversity.py* script and the observed species metric. The alpha-diversity rarefaction analysis was computed for 1000 sequences per sample for bacteria which included 71/72 samples, 320 sequences for archaea (including 70/72 samples) and 250 sequences for ciliate protozoa (including 26/36 samples). The collector's curves for the three microbial groups were visualised in SigmaPlot (2008 version 11, Systat Software, Inc., San Jose, CA, USA) by plotting the mean number + SD of OTUs observed against the number of sequences sampled.

When considering faecal samples from Groups A and B within each microbial group, bacterial phyla with relative abundances < 1% in all samples were grouped as "Other Phyla". Similarly, bacterial families or genera with relative abundances < 1% in all samples were grouped as "Other Families" or "Other Genera", respectively. The archaeal community was categorised at a mixed-taxon level, and archaeal clades with relative abundances < 1% in all samples were grouped as "Other Taxa". The ciliate protozoa community was categorised at genus level and all ciliate protozoa genera with relative abundances < 1% in all samples were grouped as "Other Genera". Sequence data generated in this study were deposited in the NCBI SRA under study accession number SRP033608.

5.3.5 Statistical analyses

Alpha-diversity was evaluated at the OTU level using the QIIME pipeline (Kuczynski *et al.*, 2012b). The sampling completeness was evaluated by using the Good's coverage estimator, which calculates the probability that a randomly selected amplicon sequence from a sample has already been sequenced (Good, 1953, Esty, 1986). Good's coverage (Good, 1953) was calculated in Excel (version 2010, Microsoft Corp., Redmond, WA, USA), and presented as mean percentage ± SD for each microbial group. Additional diversity indices (species richness, species evenness, Shannon-Wiener's diversity index, Simpson's index of

diversity) were calculated for the various levels (phylum, family and genus) for the bacterial community and a mixed-taxonomic level for the archaeal community, using the PAST software (Hammer *et al.*, 2001). The species richness was evaluated by counting the number of taxa in the community and Pielou's species evenness was calculated to explain the biodiversity in each sample by quantifying the species equality based on the distribution of relative abundances of the species in the community (ranging from 0-1, where 1 was complete evenness with least variation in the community). The Shannon-Wiener diversity index (Spellerberg and Fedor, 2003) was computed to explain the entropy, taking into account the species richness and evenness of the community, which varied from 0 for communities with a single taxon, to high values of ~4.6 for highly diverse communities. Simpson's index of diversity (1-D) (Simpson, 1949) was used to describe the diversity in a community, ranging from 0-1, with 1 indicating maximum diversity in a sample.

Beta-diversity was evaluated on a genus level for the bacterial community and a mixed-taxonomic level for the archaeal community, using the QIIME pipeline. Only microbial taxa that represented ≥ 1% of the total community, in at least one sample within each microbial group (bacteria and archaea), were included in the downstream analysis. Differences in bacterial and archaeal communities between samples were calculated using the Bray-Curtis (which takes into account the presence or absence of a species and the relative abundance) and Sørensen-Dice (which takes into account the presence or absence of a species) dissimilarity metrics. Principal coordinate analysis (PCoA) was performed in QIIME and the clustering of samples based on the first two principal coordinates was visualised in SigmaPlot. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was performed in QIIME, based on the Bray-Curtis dissimilarity matrix, to visualise the clustering of horses by diet on Days 0 and 4 of the study period, and the dendrograms were visualised in MEGA5 (version 5.2) (Tamura *et al.*, 2011).

The data generated in QIIME and PAST were imported into Excel, and re-formatted where necessary, before tests for statistical significance were conducted in STATA version 12.1 (Stata Corp, College Station, TX, USA). Non-normally distributed data are presented as median percentage and IQR throughout, and the non-parametric Kruskal-Wallis test was used to test for differences between the relative abundances of taxa identified in the faecal microbiomes of horses in Groups A and B. The level of significance used for differences between the bacterial and archaeal diversity indices on Days 0 and 4 was $P < 0.017$ after Bonferroni adjustment for multiple comparisons. The relative abundances of bacterial

communities between groups and within each group, on Days 0 and 4, were compared at multiple taxonomic levels (phylum, family and genus), with significance levels of $P < 0.004$ (phylum level) and $P < 0.001$ (family and genus levels) after Bonferroni adjustment for multiple comparisons. The relative abundances of the taxa in the archaeal communities between groups and within each group, on Days 0 and 4, were compared at a mixed-taxonomic level, with a significance level of $P < 0.008$ after Bonferroni adjustment for multiple comparisons.

Inter-group (horses in Group A and B) and intra-group (horses in the same group) variation in bacterial and archaeal community structure on Day 0 were compared using the Bray-Curtis dissimilarity matrix to create a median value for each set of comparisons. The inter-horse (between horses in Group A and B on multiple sampling days) and intra-horse (horses compared with self on multiple sampling days) variation on Days 4-21 were also compared using Bray-Curtis dissimilarity to create a median value for each set of comparisons. The non-parametric Kruskal-Wallis test was used to test differences between the median values for each set of comparisons and the level of statistical significance was $P < 0.05$.

Data for analysing the community structure of faecal microbiota (bacteria, archaea and ciliate protozoa) of pasture-fed horses are reported. The relative abundance data generated from QIIME were extracted, reformatted in excel to exclude the values for Day 0 in both Group A and B, for each microbial group, and the results presented as a median percentage and IQR. Diversity indices were calculated using the PAST software for the ciliate protozoa data for pasture-fed horses, after excluding the values for Day 0 in both Groups A and B, and presented as median and IQR.

5.4 Results

Amongst the prokaryote domains, PCR amplicons of bacteria and archaea were obtained from all 72 faecal samples. Despite repeated testing of variable dilutions of DNA template concentrations, PCR amplicons of ciliate protozoa were only obtained from half of the faecal samples (36/72), which was insufficient to adequately represent the two groups of horses on each sampling day. The results of the phylogenetic analysis for the ciliate protozoa are therefore described as part of the faecal microbiota of pasture-fed horses, but are excluded from downstream analysis involving diet-specific comparisons.

5.4.1 Metrics of pyrosequencing data for three microbial groups

The 72 faecal samples generated just under a million sequence reads (981,946) for the pooled microbial communities (bacteria, archaea and protozoa). Quality filtering and barcode mapping through the QIIME pipeline resulted in 553,715 sequences, the majority of which were, as expected from the pooling ratio, from the bacterial group (73%, 401,996), and the remaining sequences were archaea (71,403) and ciliate protozoa (80,316) (Table 5.1). Denoising of the bacterial and archaeal sequences resulted in 387,603 and 66,273 sequences respectively, and after removal of potential chimeras the number was further reduced to 387,083 and 65,639 sequences for bacteria and archaea respectively (Table 5.1). A total of 86,692 unique OTUs for bacteria and 63 unique OTUs for archaea were identified at 97% sequence similarity, from the total number of sequences obtained after chimera removal, and 36,896 unique OTUs were identified at 100% sequence similarity from all sequences for ciliate protozoa. The OTU tables for each microbial group were rarefied leaving a total number of 25,309 and 48 unique OTUs identified at 97% sequence similarity for bacteria and archaea, respectively, and 3,851 unique OTUs identified at 100% sequence similarity for the ciliate protozoa.

Within the domain Bacteria, 19 phyla were detected, which encompassed at least 93 different families and 158 different genera (Appendix C-3, Table S2A). In Groups A and B, just over half of the phyla (10/19) had relative abundances ≥ 1% in at least one sample and the remaining phyla (9/19) had relative abundances < 1% in all samples (collectively referred to as "Other Phyla"). Two thirds of the families (65/93) had relative abundances < 1% ("Other Families"), leaving a third of the families (28/93) with relative abundances ≥ 1% in at least one sample. The majority of genera (118/158) had relative abundances < 1% ("Other Genera"),

the remaining genera (40/158) had relative abundances ≥ 1% in at least on sample. Several organisms were detected, which are as yet "unclassified" in the Greengenes database. In the domain Archaea, two phyla were detected, encompassing at least five different families and 10 different clades (Appendix C-3, Table S2B). Ciliate protozoa were detected in 26 out of 72 samples and belonged to at least 15 different genera (Appendix C-3, Table S2C).

5.4.2 Rarefaction analysis and coverage of microbial diversity

Rarefaction analysis for the three microbial groups is presented in Figure 5.1. There was a plateau in the number of new OTUs detected when a minimum of approximately 320 sequences per sample and 250 sequences per sample were rarefied for the archaeal and ciliate protozoal groups, respectively. The collector's curve for the bacterial group had still not reached an asymptote when rarefied at a minimum of approximately 1,000 sequences per sample (Figure 5.1, Appendix C-4, Figure S1A,B). However, Good's coverage estimates indicated that the sampling depth had adequately captured a large part of the species diversity in all three microbial groups, with the mean coverage being 99.60 ± 0.17% for the bacterial community, 99.94 ± 0.07% for the archaeal community, and 99.90 ± 0.11% for the ciliate protozoal community.

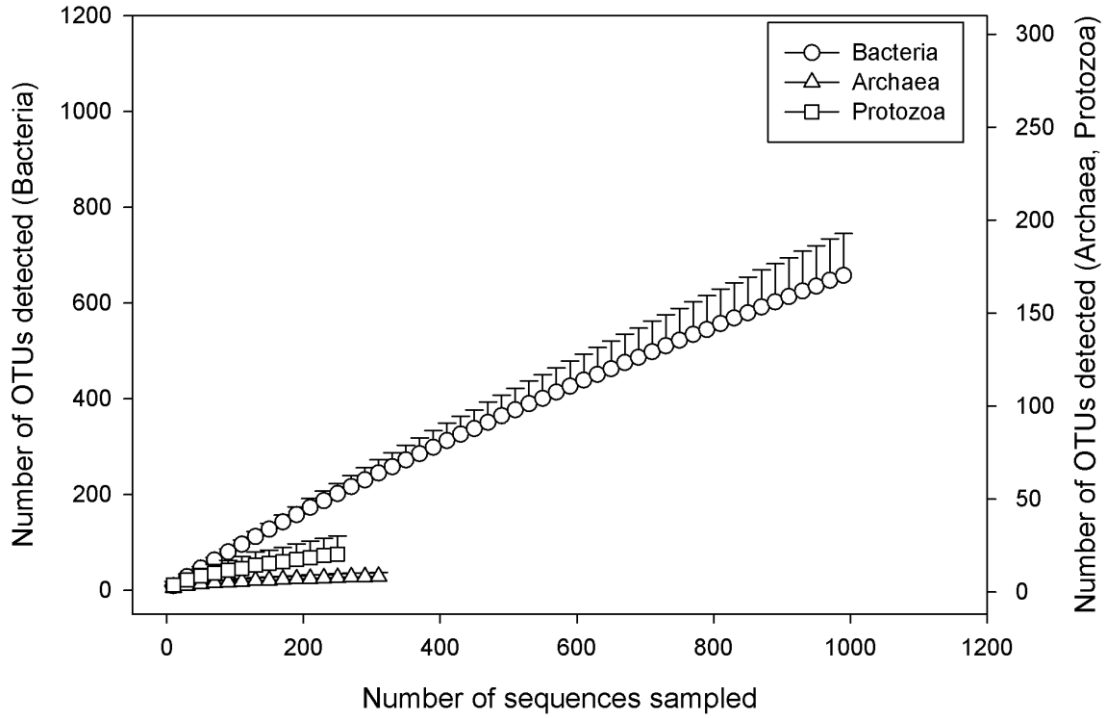
Table 5.1. Metrics of data generated by 454 GS FLX Titanium pyrosequencing of 16S and 18S rRNA gene amplicons from microbial groups present in 72 equine faecal samples.

Details	Microbial Group		
	Bacteria	**Archaea**	**Ciliate protozoa**
Sequences after quality-filtering			
Number of sequences	401,996	71,403	80,316
% of total sequences	72.6%	12.9%	14.5%
Mean number of sequences per sample	5,584	992	2171
(range)	(268-15,493)	(16-2,608)	(1-10,736)
Mean length of sequences	521.2	506.2	516.6
Mean length of sequences after removal of primers	470.8	452	483.6
Number of sequences after denoising	387,603	66,273	*
Number of sequences after chimera removal	387,083	65,639	*
Sequences after sub-sampling[§]			
Mean number of sequences per sample after sub-sampling	5,448	934	3,084
(range)	(1,333-15,006)	(322-2,438)	(252-10,736)
Number of sequences per sample (rarefied)	1000	320	250

* Not applicable (see Materials and Methods section)

[§] Samples with low number of sequences were excluded from the microbial libraries (see Materials and Methods section)

Figure 5.1. Rarefaction curves for microbial communities in faecal samples of forage-fed horses.



Legend: The rarefaction curves show the mean number (with standard deviation) of observed species against the depth of sequencing of bacterial (-o-), archaeal (-Δ-) and ciliate protozoal (-□-) communities in the equine faeces sampled in the study (n=72 faecal samples). Multiple rarefactions were calculated from the OTU tables obtained for each of the three microbial groups representing 71 out of 72 samples for bacteria (minimum of 1,000 sequence reads/sample), 70 out of 72 samples for archaea (minimum of 320 sequence reads/sample) and 26 out of 37 samples for ciliate protozoa (minimum of 250 sequence reads/sample).

5.4.3 Composition of the faecal bacterial community pre-dietary change

5.4.3.1 Diversity indices

There was a difference in the median Simpson's indices of diversity (1-D) of bacterial genera in Group A (0.80 [IQR 0.79-0.82]) and Group B (0.85 [IQR 0.84-0.85]) (P = 0.016). There were no significant differences between Group A and B for the median Shannon-Wiener (2.22 [IQR 2.07-2.33] and 2.35 [IQR 2.28-2.38]; P = 0.149) and evenness (0.31 [IQR 0.28-0.31] and 0.38 [0.31-0.32]; P = 0.423) diversity indices.

5.4.3.2 Comparison of relative abundances of taxa in the bacterial community at multiple levels

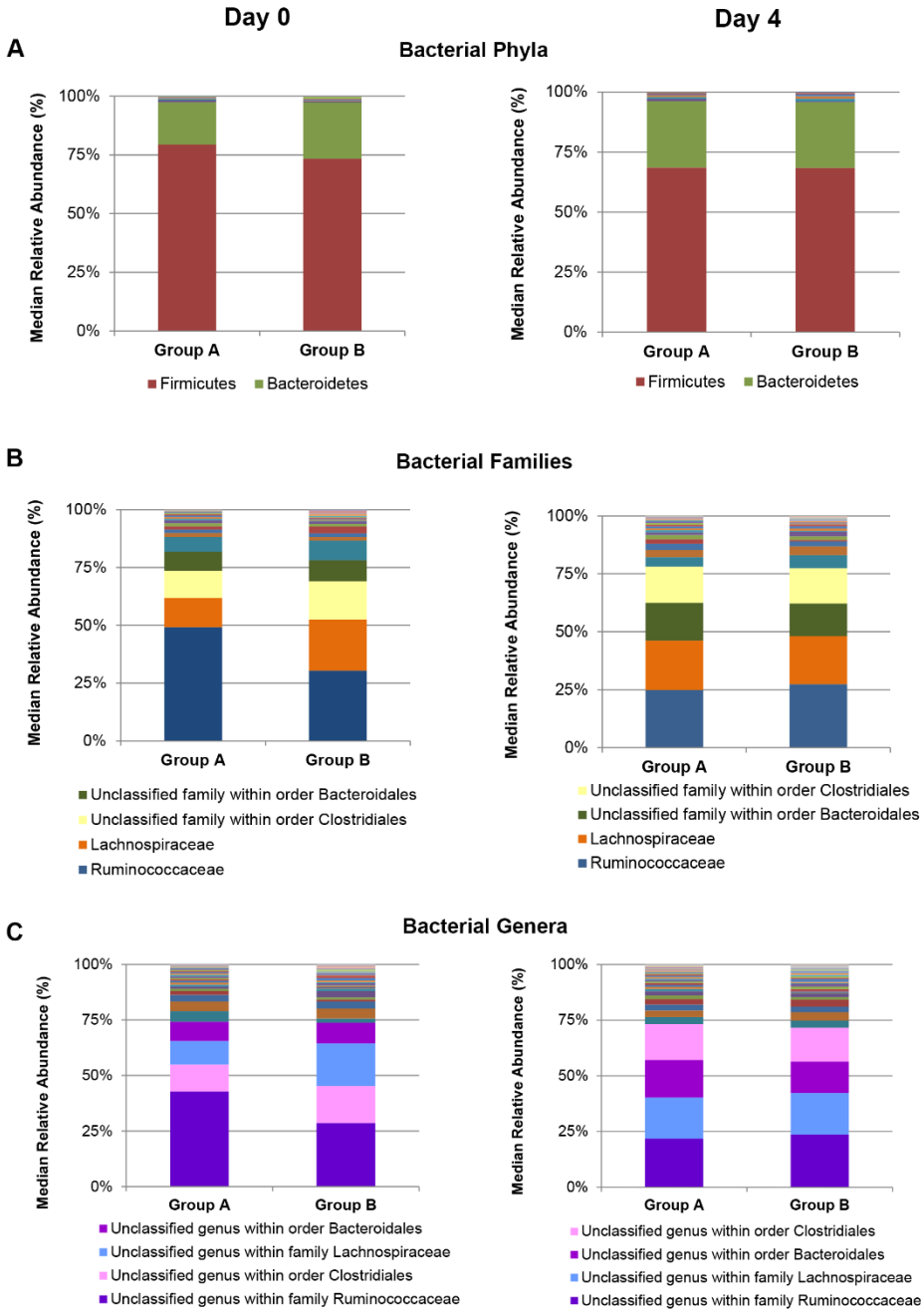
Two phyla, namely the Firmicutes and Bacteroidetes dominated the bacterial community, with median relative abundances of 80% (IQR 67-84) and 18% (IQR 13-27) in Group A horses and 74% (IQR 70-74) and 24% (22-28) in Group B horses, respectively (Figure 5.2A). There were no significant differences detected among bacterial phyla between the two diet-groups (Table 5.2). Members of the family Ruminococcaceae had the highest median relative abundance in both Group A (47% [IQR 43-53]) and Group B (30% [IQR 25-33]), followed by members of the family Lachnospiraceae (12% [IQR 8-18] in Group A; 22% [IQR 18-26] in Group B) and members of as yet unclassified families within the orders Clostridiales (11% [IQR 10-13]; 16% [IQR 15-18]) and Bacteroidales (8% [5-10]; 9% [8-13]), each in Groups A and B, respectively (Figure 5.2B). For the as yet unclassified members within the order Clostridiales, there was a difference in the median relative abundances between Groups A and B (P = 0.003).

At genus level, the highest median relative abundances were of as yet unclassified members within the family Ruminococcaceae (40% [IQR 39-41; 28% [IQR 23-29]), unclassified members within the order Clostridiales (11% [IQR 10-13]; 16% [IQR 15-18]), unclassified members within the family Lachnospiraceae (10% [IQR 7-14]; 19% [IQR 16-24]), and unclassified members within the order Bacteroidales (8% [IQR 5-10]; 9% [IQR 8-13]), each in Groups A and B, respectively (Table 5.3; Figure 5.2C). There was a difference between Groups A and B in the relative abundances of as yet unclassified members within the order Clostridiales (P = 0.003) and within the family Lachnospiraceae (P = 0.006), and the less abundant genera CF231 (P = 0.004) and BF311 (P = 0.003) (Table 5.3, Appendix C-5 Table S3A).

5.4.3.3 Beta diversity

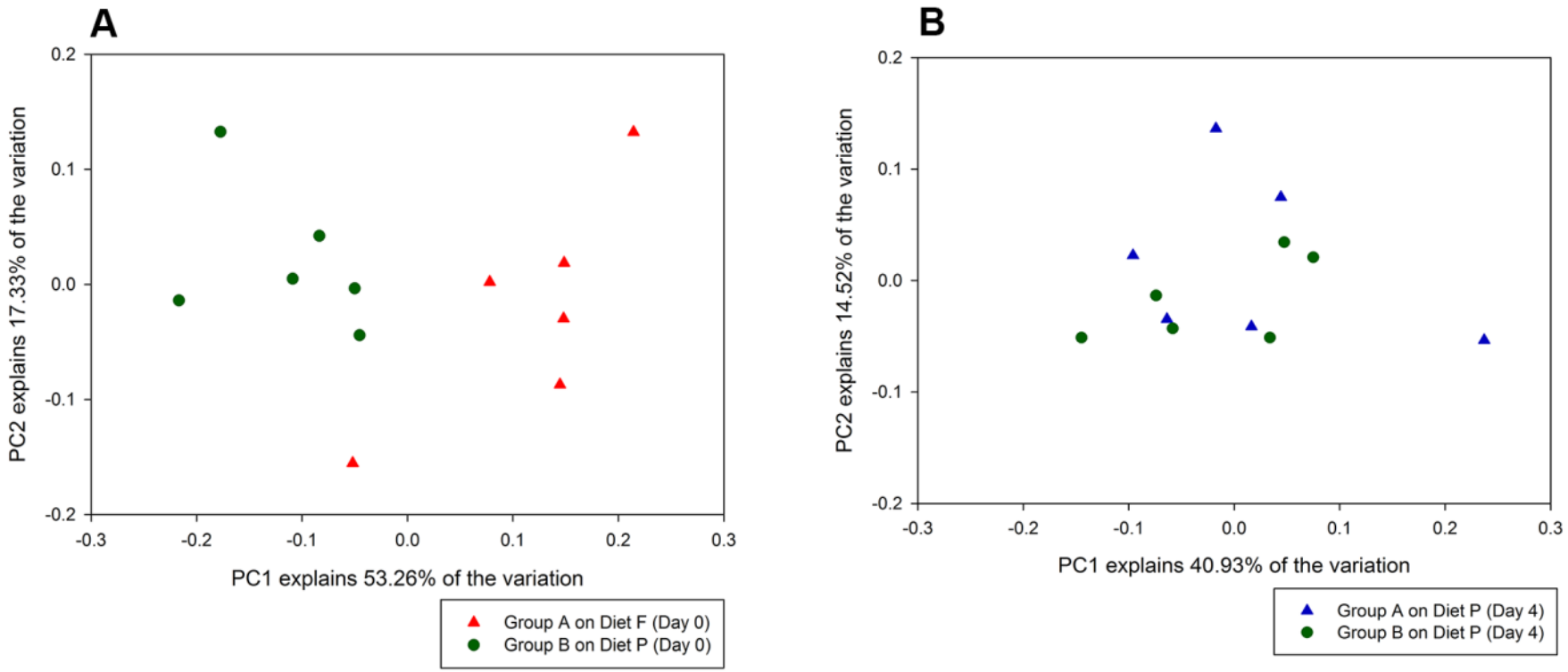
Principal coordinate analysis on genus level using Bray-Curtis dissimilarity (which takes into account presence and absence as well as relative abundance of a taxonomic group) revealed clustering of horses by dietary treatment group with more than half (53.3%) of the variation explained by PC1 and 17.3% of the variation explained by PC2 (Figure 5.3A). Similarly, UPGMA dendrograms showed distinct clusters of horses by diet (Figure 5.4). The median inter-group dissimilarity of faecal bacterial communities (0.298 [IQR 0.235-0.347]) was significantly higher (P = 0.0004) than the median intra-group dissimilarity (0.220 [IQR 0.180-0.265]) of horses on Day 0. Clustering by diet was not observed in UPGMA dendrograms using the Sørenson-Dice dissimilarity metric, which only takes into account presence and absence of a taxonomic group (Appendix C-6).

Figure 5.2. Comparison of the bacterial community structure at multiple levels.



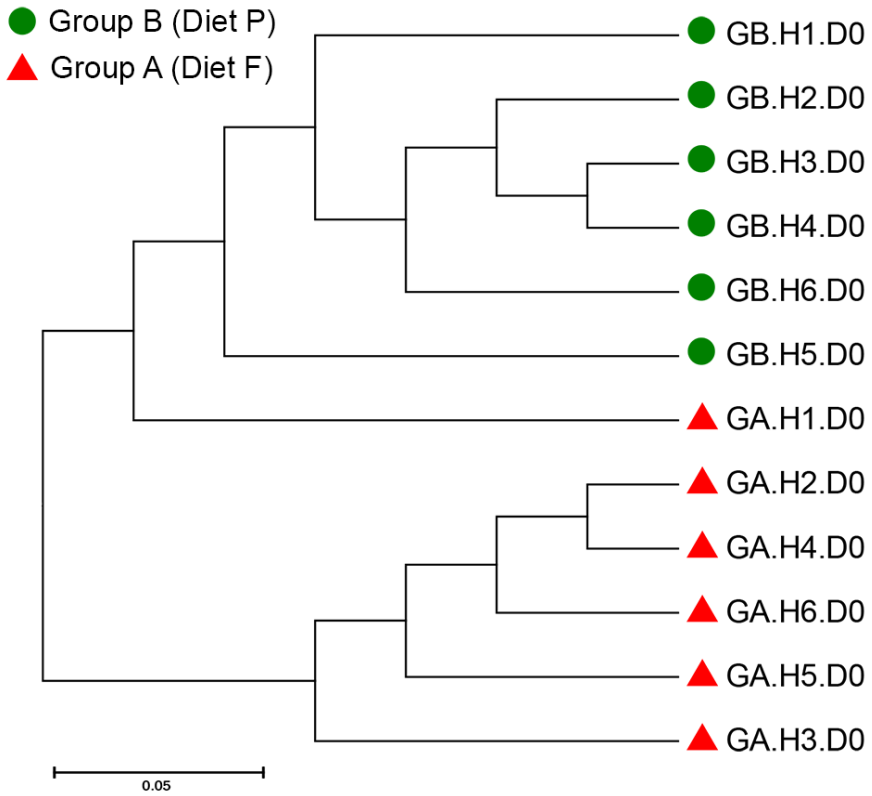
Legend: The median relative abundances of the bacterial phyla in the faeces of Group A and B horses, on Days 0 and 4, are illustrated in the figure, panel (A). Panel (B) shows the relative abundances of the bacterial families, and panel (C) shows the relative abundances of bacterial genera present in the faeces of Group A and B horses, on Days 0 and 4. The stacked bar graph is presented in ascending order of the median relative abundances of bacterial taxa for Group A horses, and the legends show the most dominant taxa in each graph (> 15% median relative abundance for the phylum level and > 8% for the family and genus levels).

Figure 5.3. Principal coordinate analysis (PCoA) of bacterial communities detected in equine faeces pre- and post-dietary change based on the Bray-Curtis dissimilarity metric.



Legend: (A) PCoA plot of faecal bacterial communities for Group A (Diet F) and Group B (Diet P) horses illustrates clustering of horses by dietary treatment group at Day 0. (B) PCoA plot of faecal bacterial communities for Group A (transition from diet F to diet P) and Group B (Diet P) horses illustrates no clustering by dietary treatment group at four days post-dietary change. Explanations for the symbols used are given in the legend.

Figure 5.4. UPGMA dendrogram based on the Bray-Curtis dissimilarity matrix on Day 0.



Legend: The Bray-Curtis dissimilarity metric takes into account the presence and absence of taxa and the relative abundances of the taxa, to generate a dissimilarity matrix for the faecal microbiota present in samples from horses on Day 0. The UPGMA dendrogram shows distinct clustering of horses (H; numbered from 1-6 per group) by group, indicating that the faecal bacterial community structure of horses on Day 0 differed between Group A (GA) and Group B (GB), which were fed Diet F and P respectively.

Table 5.2. Comparison of the relative abundances of bacterial phyla in the faeces of Group A (fed Diet F, n=6) and Group B (fed Diet P, n=6) horses on Day 0, and Day 4 (both groups fed Diet P).

	Relative abundance on Day 0					Relative abundance on Day 4				
	Group A		Group B			Group A		Group B		
Bacterial Phylum	**Median**	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**	**Median**	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**
Firmicutes	0.797	0.669-0.838	0.736	0.704-0.740	0.420	0.684	0.631-0.750	0.680	0.621-0.728	1.000
Bacteroidetes	0.181	0.131-0.273	0.240	0.216-0.278	0.260	0.276	0.222-0.298	0.272	0.223-0.335	0.870
Spirochaetes	0.006	0.004-0.007	0.003	0.002-0.004	0.140	0.004	0.002-0.005	0.008	0.004-0.010	0.060
Cyanobacteria	0.004	0.001-0.004	0.001	0.000-0.002	0.120	0.002	0.000-0.002	0.002	0.001-0.007	0.420
Fibrobacteres	0.003	0.002-0.005	0.005	0.003-0.009	0.200	0.008	0.004-0.010	0.010	0.008-0.017	0.200
Proteobacteria	0.003	0.002-0.004	0.003	0.001-0.004	0.800	0.003	0.002-0.005	0.002	0.001-0.003	0.170
Actinobacteria	0.002	0.001-0.004	0.009	0.007-0.021	0.010	0.008	0.006-0.009	0.012	0.010-0.012	0.020
Armatimonadetes	0.001	0.000-0.001	0.002	0.001-0.003	0.130	0.009	0.006-0.017	0.002	0.001-0.002	0.010
Planctomycetes	0.001	0.000-0.003	0.000	0.000-0.000	0.180	0.001	0.000-0.002	0.001	0.000-0.001	0.470
Synergistetes	0.000	0.000-0.002	0.000	0.000-0.001	0.700	0.001	0.000-0.001	0.000	0.000-0.001	0.630
Other Taxa	0.008	0.005-0.008	0.005	0.004-0.005	0.030	0.004	0.003-0.005	0.006	0.003-0.006	0.470

Legend: The bacterial phyla are listed in descending order of relative abundances for Group A horses on Day 0, and all phyla present at relative abundances of < 1% in all samples are grouped as Other Taxa.

[a] IQR – Interquartile range

[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.004$

Table 5.3. Comparison of the relative abundances of bacterial genera in the faeces of Group A (fed Diet F, n=6) and Group B (fed Diet P, n=6) horses on Day 0.

Taxonomic rank within the domain Bacteria	Relative abundance on Day 0				
	Group A		Group B		
Phylum > Class > Order > Family > Genus	Median	IQR[a]	Median	IQR[a]	P value[b]
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.395	0.387 - 0.413	0.281	0.227 - 0.293	0.016
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.112	0.103 - 0.131	0.163	0.149 - 0.181	0.003*
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.098	0.067 - 0.139	0.188	0.157 - 0.238	0.006*
Bacteroidetes > Bacteroidia > Bacteroidales >unclassified > unclassified	0.079	0.053 - 0.100	0.091	0.080 - 0.132	0.688
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.040	0.006 - 0.063	0.043	0.037 - 0.067	0.470
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.045	0.028 - 0.058	0.019	0.015 - 0.030	0.054
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > CF231	0.002	0.000 - 0.003	0.012	0.008 - 0.022	0.004*
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > BF311	0.001	0.000 - 0.001	0.010	0.009 - 0.014	0.003*

Legend: The table lists bacterial genera that were present at relative abundances of ≥ 4% in both Groups A and B, and certain genera (present at < 4% relative abundance) that were different between Groups A and B on Day 0. The taxonomic ranks are listed from Phylum to Genus in descending order of relative abundances for Group A.

[a] IQR – Interquartile range

[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$

* Differences between Groups A and B

5.4.4 Dynamic adaptation of the faecal bacterial community post-dietary change

5.4.4.1 Diversity Indices

At genus level, the median Simpson's indices of diversity (1-D) were similar between bacterial communities in Groups A (0.87 [IQR 0.86-0.89]) and B (0.86 [IQR 0.85-0.87]) on Day 4 (P = 0.425). There were no significant differences in the median Shannon-Wiener (2.50 [IQR 2.43-2.56]; 2.43 [IQR 2.42-2.49]; P = 0.335) and the evenness (0.35 [IQR 0.32-0.38]; 0.33 [IQR 0.32-0.36]; P = 0.262) diversity indices between Groups A and B, respectively. There were significant differences in the median Simpson's indices of diversity of Group A horses between Day 0 (0.80 [IQR 0.79-0.82]) and Day 4 (0.87 [IQR 0.86-0.89]); (P = 0.004). The median Shannon-Wiener diversity indices of Group A horses on Day 0 (2.22 [IQR 2.07-2.33) and Day 4 (2.47 [IQR 2.43-2.56]) were significantly different (P = 0.004). There were no differences in the evenness diversity indices of Group A horses between Days 0 and 4. There were no significant differences in the diversity indices of Group B horses on Days 0 and 4 (Appendix C-7).

5.4.4.2 Comparison of relative abundances of taxa in the bacterial community at multiple levels

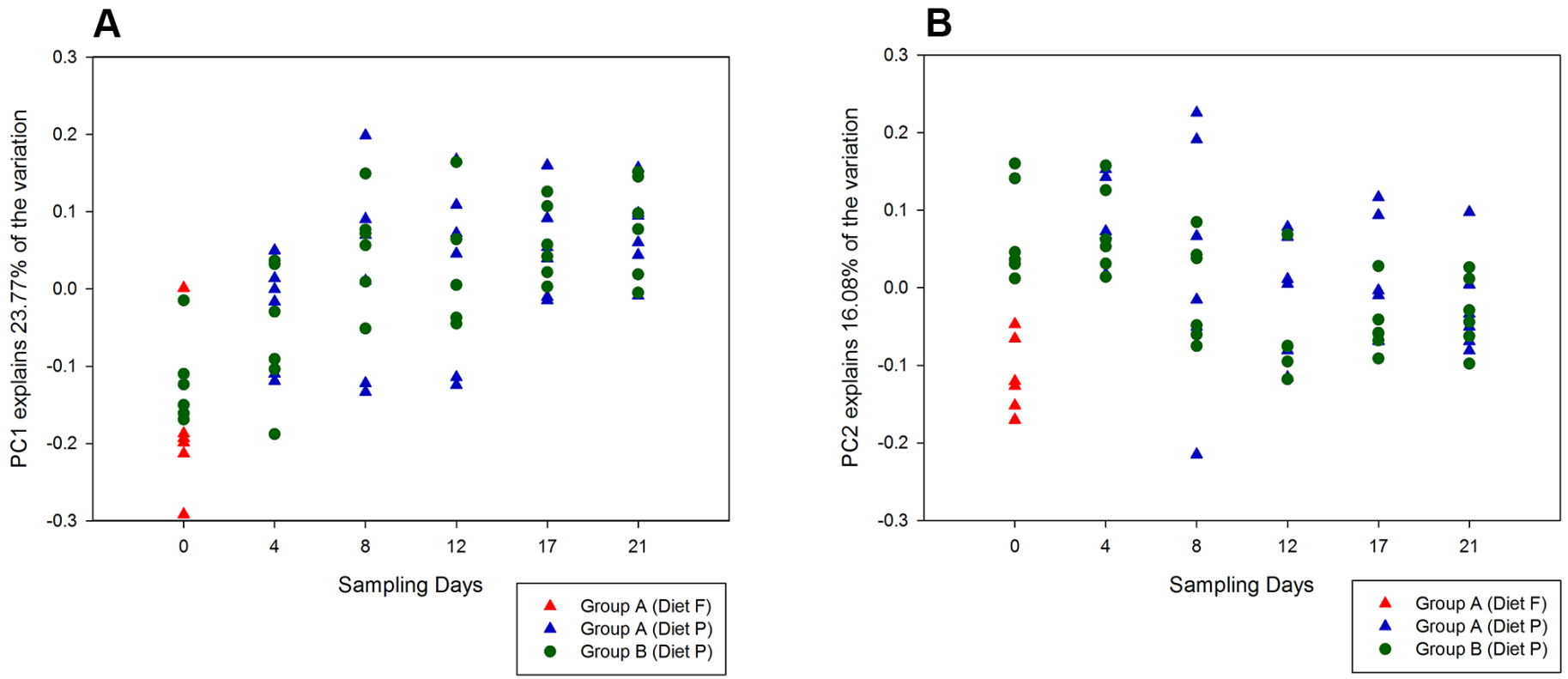
The bacterial community was dominated by two phyla, Firmicutes and Bacteroidetes, with median relative abundances of 68% (IQR 63-75) and 28% (IQR 22-30) in Group A horses and 68% (IQR 62-73) and 27% (IQR 22-34) in Group B horses, respectively (Figure 5.2A). There were no significant differences between the relative abundances of bacterial phyla detected in the faeces of Group A and B horses on Day 4 (Table 5.2). Members of the family Ruminococcaceae had the highest relative abundance in both Group A (24% [IQR 20-27]) and Group B (26% [IQR 22-33]), followed by members of the family Lachnospiraceae (21% [IQR 17-25] in Group A; 20% [IQR 19-22] in Group B), members of as yet unclassified families within the orders Bacteroidales (16% [IQR 13-18]; 13% [IQR 12-18]) and Clostridiales (15% [IQR 13-17]; 15% [IQR 13-16]), each in Groups A and B, respectively (Figure 5.2B). There were no significant differences between the relative abundances of bacterial families detected in the faeces of Groups A and B horses on Day 4 (Appendix C-8).

At genus level, the highest relative abundances in Groups A and B were of as yet unclassified members within the family Ruminococcaceae, unclassified members within the family Lachnospiraceae, and unclassified members within the orders Bacteroidales and Clostridiales (Figure 5.2C, Appendix C-5 Table S3A). Only one genus (RFN20, belonging to the phylum Firmicutes, family Erysipelotrichaceae), present at a low abundance (< 2%), showed a difference in relative abundance between Groups A and B on Day 4 (P = 0.004; Appendix C-5 Table S3A). When comparing the faecal bacterial community of Group A horses, the relative abundances of three bacterial genera (*Pseudoramibacter /Eubacterium* and as yet unclassified members within the family Ruminococcaceae and within the order RB046 of the phylum Armatimonadetes) differed between Day 0 and Day 4 (Table 5.4, Appendix C-5 Table S3B). There were no significant differences between the bacterial genera detected in the faeces of Group B horses that were grazing pasture on both Days 0 and 4 (Appendix C-5 Table S3C).

5.4.4.3 Beta diversity

On Day 4, bacterial communities did not cluster by diet-group (Figure 5.3B). Furthermore, there was no clustering by group from Day 4 through to Day 21 (Figure 5.5). Diet-specific clusters were observed between the horses fed Diet F (Group A horses on Day 0) and those fed pasture (Groups A horses on Days 4-21 and Group B horses on Days 0-21), (Appendic C-9 Figure S4). Inter- and intra-horse variation across all horses on pasture (Days 4-21 of both groups) had a median dissimilarity of 0.236 (IQR 0.189-0.291) and 0.222 (IQR 0.176-0.276), respectively (P = 0.044).

Figure 5.5. Principal coordinate analysis (PCoA) of bacterial communities detected in equine faeces over 3-weeks based on the Bray-Curtis dissimilarity metric.



Legend: The plots illustrate the structure of the faecal bacterial communities in horses of Groups A and B on six sampling time-points over a period of three weeks. The plots show group-wise clustering of horses fed Diet F and Diet P on Day 0, and no clustering when horses were fed pasture (Diet P) from Days 4-21, with 24% variation explained on PC1 (A) and 16% variation explained on PC2 (B).

Table 5.4. Comparison of the relative abundances of bacterial genera in the faeces of Group A horses on Day 0 (fed Diet F, n=6) and Day 4 (fed Diet P, n=6).

Taxonomic rank within the domain Bacteria	**Relative abundance of Group A horses**				
Phylum > Class > Order > Family > Genus	**Day 0**		**Day 4**		
	Median	**IQR[a]**	**Median**	**IQR[a]**	***P* value[b]**
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.395	0.387 - 0.413	0.205	0.171 - 0.220	0.004*
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.112	0.103 - 0.131	0.151	0.130 - 0.167	0.078
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.098	0.067 - 0.139	0.173	0.151 - 0.195	0.025
Bacteroidetes > Bacteroidia > Bacteroidales >unclassified > unclassified	0.079	0.053 - 0.100	0.158	0.132 - 0.177	0.109
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.045	0.028 - 0.058	0.025	0.018 - 0.032	0.146
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.040	0.006 - 0.063	0.023	0.017 - 0.025	0.749
Firmicutes > Clostridia > Clostridiales > Eubacteriaceae > *Pseudoramibacter_Eubacterium*	0.001	0.000 - 0.002	0.006	0.004 - 0.007	0.004*
Armatimonadetes > SJA-176 > RB046 > unclassified > unclassified	0.001	0.000 - 0.001	0.009	0.006 - 0.017	0.005*

Legend: The table lists bacterial genera that were present at relative abundances of ≥ 4% in the faeces of Group A horses, and certain genera (present at < 4% relative abundance) that were different between Days 0 and 4. The taxonomic ranks are listed from Phylum to Genus in descending order of relative abundances for Day 0.

[a] IQR – Interquartile range

[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$

* Differences between Days 0 and 4

5.4.5 Composition of the faecal archaeal community pre- and post-dietary change

The median Simpson's indices of diversity were similar for both groups A (0.48 [IQR 0.19-0.55]; 0.42 [IQR 0.40-0.48]) and B (0.42 [IQR 0.36-0.46]; 0.45 [IQR 0.41-0.49]), on Days 0 and 4, respectively. The median Shannon-Wiener diversity indices for Group A (0.81 [IQR 0.44-0.95]; 0.70 [IQR 0.67-0.70]) and Group B (0.68 [IQR 0.60-0.72]; 0.70 [IQR 0.68-0.75]), and the evenness diversity indices for Group A (0.5 [IQR 0.31-0.61]; 0.59 [IQR 0.44-0.69]) and Group B (0.51 [IQR 0.45-0.68]; 0.54 [IQR 0.49-0.68]) on Days 0 and 4 respectively, were also similar. There were no significant differences in the diversity indices of the faecal archaeal community between Group A horses on Days 0 and 4, between Group B horses on Days 0 and 4, and between Group A and B horses on either Day 0 or Day 4 (Appendix C-10).

Over half (6/10) of the archaeal clades in Groups A and B were present at a relative abundance of ≥ 1% in at least one sample, and the remaining clades were grouped as "Other Taxa". The archaeal community was dominated by two clades, *Methanocorpusculum* and relatives and *Methanobrevibacter ruminantium* and relatives in both groups, on both Day 0 and Day 4 (Appendix C-11 Figure S2, Appendix C-12 Table S4A,B). There were no significant differences in the median relative abundances of archaeal taxa between Group A horses on Days 0 and 4 (Appendix C-12 Table S4A), and Group B horses on Days 0 and 4 (Appendix C-12 Table S4B). There were no significant differences in the median relative abundances of archaeal taxa between Groups A and B on Day 0 or Day 4 (Table 5.5, Appendic C-12 S4C). Principal coordinate analysis did not show group-wise clustering of horses on Day 0 or on Days 4-21 (Appendix C-13 Figure S3). There was no significant difference in the inter-group or intra-group dissimilarity on Day 0 or on Days 4-21 (P = 0.987).

Table 5.5. Comparison of the relative abundances of archaeal taxa (clade-level) in the faeces of Group A (fed Diet F, n=6) and Group B (fed Diet P, n=6) horses on Day 0.

Taxonomic rank under the domain Archaea	**Relative abundance on Day 0**				
Phylum > Class > Order > Genus / Clade	**Group A**		**Group B**		
	Median	**IQR[a]**	**Median**	**IQR[a]**	***P* value[b]**
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_ruminantium*_and_relatives	0.630	0.413 - 0.897	0.663	0.569 - 0.769	0.710
Euryarchaeota > Methanomicrobia > Methanomicrobiales > *Genera_incertae_sedis* > *Methanocorpusculum*_and_relatives	0.177	0.056 - 0.309	0.319	0.219 - 0.431	0.200
Euryarchaeota > Thermoplasmata > Thermoplasmatales > Rumen Cluster C_and_relatives	0.016	0.003 - 0.031	0.016	0.009 - 0.038	0.850
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_gottschalkii*_and_relatives	0.014	0.000 - 0.028	0.000	0.000 - 0.003	0.120
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanosphaera*	0.011	0.003 - 0.025	0.000	0.000 - 0.000	0.030
Other Taxa	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.360

[a] IQR – Interquartile range
[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.008$

5.4.6 Composition of the faecal microbiota of horses maintained exclusively on pasture

The faecal microbiota of all horses (Groups A and B) grazing on pasture from Days 4-21 comprised of a diversity of bacteria, archaea and ciliate protozoa. The highest median relative abundances of bacterial taxa were of unclassified members within the family Ruminococcaceae (22% [IQR 17-27]), the order Bacteroidales (21% [IQR 17-25]), the order Clostridiales (12% [IQR 10-14]) and within the family Lachnospiraceae (11% [IQR 10-15]), which together contributed two-thirds (66%) of the bacterial community. The bacterial genera detected at > 1% relative abundance were *Bacteroides, Blautia, BF311, CF231, Clostridium, Coprococcus, Fibrabacter, Mogibacterium, Oscillospira, Paludibacter, Parabacteroides, Prevotella, Pseudoramibacter/eubacterium,* p-75-a5*, RFN20, Ruminococcus, Treponema* and *YRC22.* The microbiota of pasture-fed horses included 10 archaeal genera and their clades, six of which were present at ≥ 1% in at least one sample. The remaining four genera and clades (including *Methanobacterium, Sulfolobus* and relatives, *Methanobrevibacter arboriphilus* and relatives and *Methanosarcina* and relatives) were present at < 1% relative abundance (collectively referred to as "Other Taxa"). The highest median relative abundances were of two clades, *Methanocorpusculum* and relatives (77% [IQR 58-90]) and *Methanobrevibacter ruminantium* and relatives (17% [IQR 8-39]), which together contributed 94% of the archaeal community in the faeces of pasture-fed horses. The remaining 6% was of the clade Rumen Cluster C and relatives (median relative abundance 3% [IQR 2-4]) and the genera *Methanosphera, Methanimicrococcus* and Other Taxa, each at <1% median relative abundances.

Within the ciliate protozoa community (Appendix C-3 Table S2C), 15 genera under the class Litostomatea were identified as part of the faecal microbiota of pasture-fed horses. These included nine genera under the sub-class Trichostomatia present at ≥ 1% in at least one sample, and at least six other genera under the sub-classes Trichostomatia and Haptoria that were present at < 1% relative abundance (collectively referred to as "Other Ciliate Genera"). The median Simpson's index of diversity (1-D) for the ciliate community of pasture-fed horses was 0.66 (IQR 0.48-0.73), and the diversity indices for Shannon-Wiener and evenness were 1.4 (IQR 0.9-1.5) and 0.49 (IQR 0.40-0.61), respectively. The highest median relative abundance was represented by the genus *Spirodinium* (24% [IQR 7-41]), followed by the genera *Triadinium* (9% [IQR 4-22]) and *Cochliatoxum* (6% [IQR 1-11]). The other genera

identified were *Blepharocorys*, *Bundleia*, *Cycloposthium* (each at ~4% median relative abundance) and *Isotricha*, *Polydiniella*, and the group of Other Ciliate Genera (including *Didinium*, *Epiphyllum*, *Pelagodileptus*, *Diplodinium*, *Entodinium* and *Eremoplastron*) each at < 1% median relative abundance.

5.5 Discussion

The present study investigated changes in the faecal microbiota via pyrosequencing of 16S rRNA gene amplicons derived from microbes present in the faeces of two groups of yearling horses. The results demonstrated dietary effects on the bacterial communities in the faeces, with changes occurring in their relative abundances within four days following dietary transition.

In the present study, group-wise clustering of bacterial community structures on Day 0 was observed only by using the Bray-Curtis dissimilarity metric (Figure 5.3A and 5.4) and not the Sørensen-Dice dissimilarity metric (Appendix C-6), indicating that the differences in community structure observed in the PCoA plots were due to differences in the relative abundances of the bacterial genera, and not the presence or absence of certain genera in the faecal samples from horses fed either of the two diets. Due to PCR bias (Lee *et al.*, 2012), there are some limitations in the use of sequencing techniques to compare the true diversity of microbial communities, as there is a possibility that true abundances in an ecosystem may differ from those detected. However, based on current literature, it appears fair to compare relative abundances of microbial populations in samples that have been processed equally. Therefore, it is reasonable to conclude that the initial differences observed between the faecal microbiota of horses in the two groups of the present experiment were linked to nutritional / dietary factors, given the convergence of the microbial community structure once on similar pasture diets.

The median Simpson's index of diversity for the bacterial community in the faeces of horses fed the ensiled conserved forage-grain diet differed significantly from that of horses on pasture, and upon dietary transition, the diversity increased rapidly from 0.80 to 0.87 within four days of feeding on pasture. A high diversity in the microbiota may be a natural evolutionary strategy for survival of a cursorial herbivorous browser, providing the opportunity to rapidly respond to varying quantities and types of forage available at different time-points. Annual and seasonal variation has been shown to occur in the growth and

composition of the rye-clover mix pasture, and the water-soluble carbohydrate content in the grass may change diurnally (Litherland *et al.*, 2002). The moderate fluctuation in the bacterial community structure observed in the horses maintained on pasture in the present study may be a result of changes in the composition of pasture and warrants further investigation (Figure 5.5).

The transition, from different bacterial community structures at the start of the experiment to similar community structures when both groups were maintained on pasture, occurred rapidly within a four-day period. This was a shorter transition time than the six days post-dietary change in faecal samples previously reported (van den Berg *et al.*, 2013), and may be due to the use of a more sensitive technique in the present study. The dietary challenge in the present trial was moderate and primarily forage-based. The rapid change observed in the structure of the faecal bacterial community when horses were transitioned from feeding on an ensiled conserved forage-grain diet to pasture, and the subtle changes in the bacterial community structure observed when on pasture, emphasizes the sensitivity of the microbiota to changes in dietary substrate. Given the results of the present study, it may be suggested that the basal microbiota that inhabits the horse's gastrointestinal tract is highly diverse and primed to respond to acute changes in diet. Hence, a shorter more intense sampling period around the time of dietary transition should be considered for future studies on the faecal microbiota of horses.

Bacteria are reported to form the largest proportion of the equine hindgut and faecal microbiomes (Dougal *et al.*, 2012) with the bacterial community dominated by the phyla Firmicutes and Bacteroidetes (Costa *et al.*, 2012, Dougal *et al.*, 2013), which was also observed in the present study. At Day 0, there were no differences between Group A and Group B in the beta-diversity of the bacterial communities at the phylum level; however, there were differences at higher taxonomic resolution (at family and genus levels) (Figure 5.2). This finding of differences in the beta-diversity at family and genus levels is in contrast to the findings of other studies where a mixed population of horses were fed a variety of diets (Costa *et al.*, 2012, Steelman *et al.*, 2012, O' Donnell *et al.*, 2013), and may be due to the higher resolution possible with the use of the next generation sequencing technique.

The number of unclassified bacterial genera (68) detected in the present study demonstrates the paucity of knowledge on the composition of the microbiota (Appendix C-3 Table S2A). Therefore, further cultivation and non-cultivation based studies in various

populations of horses are required to evaluate the abundance and occurrence of the as yet unclassified organisms and to understand their functional role in hindgut microbial fermentation.

Lactic acid bacteria of the genera *Lactobacillus* and *Streptococcus* are potentially associated with grain diets and have been widely reported within the literature (Drogoul *et al.*, 2001, Costa and Weese, 2012). These genera have been implicated in the development of gastrointestinal disturbances and laminitis (Elliott and Bailey, 2006, Milinovich *et al.*, 2006, Costa *et al.*, 2012). In the present study, the genus *Lactobacillus* was present at < 1% relative abundance in the faecal microbiomes, whereas the genus *Streptococcus* was not detected in either group of horses, not even at relative abundances of < 1%. Although a higher abundance of these genera may be detected in proximal regions of the hindgut than in the faeces, our findings are in contrast to many other reports in the literature (analysing caecal, colonic and faecal samples) when horses were fed grain: forage combinations or different types of forages (Julliand *et al.*, 2001, Muhonen *et al.*, 2010, O' Donnell *et al.*, 2013, van den Berg *et al.*, 2013). None of the horses in the present study showed signs of colic, laminitis, or digestive disturbances throughout the study period, or the three weeks prior to Day 0. Given that grain diets are associated with shifts in microbial populations (Fernando *et al.*, 2010), the low abundance or non-detection of the lactic acid bacteria in the present study could be related to the dietary history of the yearling horses, wherein the management was primarily pasture-based and large quantities of grain had not been offered. There are indications that providing good quality pasture and increased activity may play a role in buffering the negative effects of grain supplementation on the hindgut microbiota (Rogers *et al.*, 2004). It is not clear what quantity of grain or soluble carbohydrate content in pasture results in shifts in hindgut microbial populations, and this requires further investigation.

The present study targeted multiple domains of microorganisms and provided new information about the influence of diet on the structure of the Bacteria and other members of the eHGM, such as the archaea and ciliate protozoa. The diversity of the archaeal community was limited in the present study (Figure 5.1), and similar to previous studies (Fernando *et al.*, 2010, Kittelmann *et al.*, 2013), with sequence reads being assigned to only 10 different archaeal clades. The presence of the two archaeal clades (*Methanobrevibacter ruminantium* and relatives and *Methanocorpusculum* and relatives) in equine faeces has previously been reported (O' Donnell *et al.*, 2013, St-Pierre and Wright, 2013), and these two clades dominated the archaeal microbiome of all horses in the present study (Appendix C-11 Figure S2).

Analysis of the archaeal community of the horses did not show clustering by diet-group and none of the archaeal taxa detected were significantly more or less abundant in either of the treatment groups (Appendix C-13 Figure S3). These results indicate that the archaeal community structure was not influenced by the change in diet. This finding is aligned with the physiology of the archaeal organisms, which rely on the hydrogen produced by cellulolytic and other bacterial populations reported in the present study and only indirectly on the type of dietary substrate available to the host. However, this does not preclude that by using greater sample size or sequencing depth, impacts on the archaeal community may have been detected.

In the present study, at least 15 different genera of ciliate protozoa were detected in the faeces of pasture-fed horses (Appendix C-3 Table S2C), of which the genera *Spirodinium* and *Triadinium* were the most abundant. In contrast, previous microscopy studies have reported that the highest percentage composition of ciliates identified in the faeces of horses, were of the genera *Bundleia*, *Blepharocorys*, and *Polymorphella* (Gürelli and Göçmen, 2011, Göçmen *et al.*, 2012, Gürelli and Göçmen, 2012). The faecal samples in these studies were collected from racehorses that were fed a mixed diet comprised of grain and forages, or from horses with an unknown dietary history; whereas the faecal samples in the present study were from horses fed exclusively on pasture. Faecal microscopic examination was not used in the present study and the ciliate protozoa were amplified from only 26/72 faecal samples. Hence, it was not possible to determine whether there were differences between the ciliate communities of the horses fed different forage-based diets on Day 0 of the study, which may require further investigation in a large number of samples.

The horses used in the present study were of similar age, sex, height, weight and body condition, and were maintained as cohorts in paddocks on the same farm. This is in contrast to most other studies that sampled heterogeneous populations of horses that differed in their feeding and management (Costa *et al.*, 2012, Dougal *et al.*, 2013, O' Donnell *et al.*, 2013). The study was not designed to separate the effects of housing from the dietary effects between Groups A and B prior to Day 0, and the change from a loose-box environment to pasture may have influenced the results. However, faecal microbiota are more likely to be influenced by acute changes in diet than more subtle changes in housing. Although the sample size for each group was small (n=6), it was similar to sample sizes used in previous 454 pyrosequencing-based studies investigating the hindgut microbiome (Costa *et al.*, 2012, O' Donnell *et al.*, 2013).

The faecal samples in the present study were collected carefully from the faecal mass and snap frozen within two minutes of defecation, thereby limiting the possibility of environmental contamination. A number of studies have demonstrated a strong inter-relationship between the microbial communities in the distal region of the hindgut (colon) and faeces (Hastie *et al.*, 2008, Murray, 2010, Dougal *et al.*, 2013). This inter-relationship of microbial communities along the hindgut was reported to be greatest between samples from the right dorsal colon and the rectum / faeces (Dougal *et al.*, 2012). Therefore, it is reasonable to suggest that the changes observed in the microbial populations of the faecal samples in the present study may be representative of changes occurring in the distal regions of the hindgut. The inter-variation between Groups A and B was higher than intra-variation, as demonstrated by the clustering of horses by treatment group on Day 0 (Figure 5.4), which allowed the identification of differences between the faecal microbiota of horses fed two different forage-based diets using a sample size of six horses per group.

5.6 Conclusions

The findings of the present study indicate that the faecal bacterial community of yearling horses is highly diverse and the relative abundances of individual taxa change rapidly in response to changes in diet. The faecal microbiota of horses on a conserved forage-grain diet were similar to that of horses fed pasture in terms of species richness and diversity, and the structure of the archaeal communities, but differed significantly in terms of the relative abundances of distinct bacterial families and genera. It is possible that daily changes in pasture composition affect the faecal microbiota of horses and this requires further investigation.

5.7 Acknowledgments

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Manawatu, New Zealand) and the horse feed for the preliminary study provided by Fiber Fresh Feeds Ltd. (Reporoa, New Zealand), for which we are grateful.

Appendix C

This appendix provides supplementary information on the tables and figures cited in Chapter 5.

C-1. An illustration of the loose-boxes and the paddock where the yearling horses were maintained during the study period.

C-2. Table S1. Dry matter content and nutrient composition of the diets.

C-3. Table S2. The faecal microbiome of New Zealand Thoroughbred yearling horses fed Diet F (ensiled-forage-grain diet) and Diet P (rye-clover pasture) during the study.

C-4. Figure S1. Rarefaction curves for bacterial communities in the faeces of individual horses in Groups A and B at two time-points.

C-5. Table S3. Comparison of the relative abundances of bacterial taxa (genus-level) in the faeces of Group A and B horses on Days 0 and 4 of the study.

C-6. UPGMA dendrograme based on the Sørensen-Dice dissimilarity metric of faecal sample on Day 0.

C-7. Diversity indices for bacterial taxa in the faeces of Group B horses.

C-8. Comparison of relative abundances of bacterial families in the faeces of Groups A and B horses on Day 4.

C-9. Figure S4. 3-dimensional plot of bacterial communities in all 72 samples.

C-10. Diversity indices for archaeal taxa in the faeces of Group A and B horses.

C-11. Figure S2. Relative abundance of archaeal clades in the faecal microbial community of horses.

C-12. Table S4. Comparison of the relative abundances of archaeal taxa (clade-level) in the faeces of Group A and B horses on Days 0 and 4 of the study.

C-13. Figure S3. Principal coordinate plot for data on the archaeal community structure in the faeces of horses across all sampling days based on Bray-Curtis dissimilarity.

C-1. An illustration of the loose-boxes (A) and the paddock (B) where the yearling horses were maintained during the study period.

C-2. Table S1. Dry matter content and nutrient composition of the diets.

A)

	Diet F
	Group A horses
Dry matter, g/100g	59.30
Crude protein, g/100g	11.70
Crude fibre, g/100g	14.80
Crude fat, g/100g	3.00
Calcium, g/100g	1.00
Phosphorus, g/100g	0.52
Selenium, mg/100g	0.03
Digestible energy (MJ/kg)	11.98

Legend: Nutrient composition of the ensiled lucerne and cracked maize feed (Diet F) that was provided to the horses of Group A prior to Day 0. The nutrient composition is given on a Dry Matter basis, as provided by the manufacturers (Fiber Fresh Feeds Ltd., Reporoa, New Zealand).

B)

	Diet P	
	Group A Horses	Group B Horses
Dry Matter %	29.5	28.5
Ash %	7.9	7.2
Fat %	2.2	2.3
Crude Protein %	13.2	11.1
Neutral Detergent Fibre (NDF) %	52.8	52.3
Acid Detergent Fibre (ADF) %	25.9	27.0
Lignin %	3.1	3.2
Starch %	1.1	0.8
Gross Energy kJ/g	18.0	18.0

Legend: Nutrient composition (Dry Matter basis) of the standard New Zealand rye-clover mixed pasture (Diet P) that was available to Group B horses prior to Day 0 of the study, and Group A and B horses during the study (Days 0-21).

C-3. Table S2. The faecal microbiome of New Zealand Thoroughbred yearling horses fed Diet F (ensiled-forage-grain diet) and Diet P (rye-clover pasture) during the study.

The bacterial, archaeal, and ciliate protozoal taxa identified in the faeces of horses in the present study are listed in the table according to the taxonomic ranks assigned using the Greengenes database (version gg_13_5).

Table S2 A. The faecal bacterial community

Domain	Phylum	Class	Order	Family	Genus	Species
Bacteria	Actinobacteria	Acidimicrobia	Acidimicrobiales	unclassified	unclassified	unclassified
		Actinobacteria	Actinomycetales	Microbacteriaceae	unclassified	unclassified
					Agromyces	unclassified
					Curtobacterium	unclassified
					Microbacterium	unclassified
					Mycetocola	unclassified
					Salinibacterium	unclassified
				Micrococcaceae	unclassified	unclassified
					Arthrobacter	unclassified
				Mycobacteriaceae	*Mycobacterium*	unclassified
				Nocardiaceae	*Rhodococcus*	unclassified
					Rhodococcus	*R. fascians*
				Nocardioidaceae	unclassified	unclassified
				Streptomycetaceae	unclassified	unclassified
					Streptomyces	unclassified
				Williamsiaceae	*Williamsia*	unclassified
		Coriobacteriia	Coriobacteriales	Coriobacteriaceae	unclassified	unclassified
					Adlercreutzia	unclassified
					Eggerthella	unclassified
		OPB41	unclassified	unclassified	unclassified	unclassified
	Armatimonadetes	SJA-176	RB046	unclassified	unclassified	unclassified
	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified	unclassified	unclassified
				BS11	unclassified	unclassified
				Bacteroidaceae	unclassified	unclassified
					5-7N15	unclassified
					BF311	unclassified
					Bacteroides	unclassified

Domain	Phylum	Class	Order	Family	Genus	Species
						B. coprophilus
						B. coprosuis
						B. plebeius
						B. uniformis
				Marinilabiaceae	unclassified	unclassified
				Porphyromonadaceae	*Paludibacter*	unclassified
					Parabacteroides	unclassified
					Parabacteroides	*P. distasonis*
				Prevotellaceae	unclassified	unclassified
					Prevotella	unclassified
						P. copri
						P. melaninogenica
						P. stercorea
				RF16	unclassified	unclassified
				Rikenellaceae	unclassified	unclassified
					PW3	unclassified
				S24-7	unclassified	unclassified
				[Barnesiellaceae]	unclassified	unclassified
				[Odoribacteraceae]	*Butyricimonas*	unclassified
				[Paraprevotellaceae]	unclassified	unclassified
					CF231	unclassified
					Paraprevotella	unclassified
					YRC22	unclassified
					[Prevotella]	unclassified
				p-2534-18B5	unclassified	unclassified
		[Saprospirae]	[Saprospirales]	Chitinophagaceae	unclassified	unclassified
	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231	unclassified
			GCA004	unclassified	unclassified	unclassified
		Dehalococcoidetes	Dehalococcoidales	Dehalococcoidaceae	unclassified	unclassified
		Thermomicrobia	JG30-KF-CM45	unclassified	unclassified	unclassified
	Cyanobacteria	4C0d-2	YS2	unclassified	unclassified	unclassified
		Chloroplast	Streptophyta	unclassified	unclassified	unclassified
	Elusimicrobia	Elusimicrobia	Elusimicrobiales	Elusimicrobiaceae	unclassified	unclassified
			unclassified	unclassified	*Elusimicrobium*	unclassified
		Endomicrobia	unclassified	unclassified	unclassified	unclassified
	Fibrobacteres	Fibrobacteria	Fibrobacterales	Fibrobacteraceae	*Fibrobacter*	*F. succinogenes*
	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. longiquaesitum*

Domain	Phylum	Class	Order	Family	Genus	Species
				Paenibacillaceae	*Paenibacillus*	unclassified
				Planococcaceae	*Sporosarcina*	unclassified
			Lactobacillales	Aerococcaceae	unclassified	unclassified
				Carnobacteriaceae	*Trichococcus*	unclassified
				Lactobacillaceae	*Lactobacillus*	unclassified
						L. salivarius
		Clostridia	unclassified	unclassified	unclassified	unclassified
			Clostridiales	unclassified	unclassified	unclassified
				Caldicoprobacteraceae	*Caldicoprobacter*	unclassified
				Christensenellaceae	unclassified	unclassified
				Clostridiaceae	unclassified	unclassified
					02d06	unclassified
					Clostridium	unclassified
						C. butyricum
					Geosporobacter/Thermotalea	unclassified
					Sarcina	unclassified
				Dehalobacteriaceae	unclassified	unclassified
				EtOH8	unclassified	unclassified
				Eubacteriaceae	*Anaerofustis*	unclassified
					Pseudoramibacter/Eubacterium	unclassified
				Lachnospiraceae	unclassified	unclassified
					Anaerostipes	unclassified
					Blautia	unclassified
					Blautia	*B. producta*
					Butyrivibrio	unclassified
					Coprococcus	unclassified
						C. catus
						C. eutactus
					Dorea	unclassified
						D. formicigenerans
					Epulopiscium	unclassified
					Lachnobacterium	unclassified
					Lachnospira	unclassified
					Moryella	unclassified
					Pseudobutyrivibrio	unclassified
					Roseburia	unclassified
						R. faecis

Domain	Phylum	Class	Order	Family	Genus	Species
					[Ruminococcus]	unclassified
						R. gnavus
						R. torques
				Peptococcaceae	unclassified	unclassified
					Desulfotomaculum	unclassified
					rc4-4	unclassified
				Peptostreptococcaceae	unclassified	unclassified
				Ruminococcaceae	unclassified	unclassified
					Ethanoligenens	unclassified
					Faecalibacterium	unclassified
						F. prausnitzii
					Oscillospira	unclassified
					Ruminococcus	unclassified
						R. bromii
						R. flavefaciens
				Syntrophomonadaceae	*Syntrophomonas*	unclassified
				Veillonellaceae	unclassified	unclassified
					Anaerovibrio	unclassified
					Phascolarctobacterium	unclassified
					Selenomonas	unclassified
					Succiniclasticum	unclassified
				[Acidaminobacteraceae]	WH1-8	unclassified
				[Mogibacteriaceae]	unclassified	unclassified
					Anaerovorax	unclassified
					Mogibacterium	unclassified
		Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	unclassified	unclassified
					Bulleidia	unclassified
						p-1630-c5
					Coprobacillus	unclassified
					L7A E11	unclassified
					RFN20	unclassified
					Sharpea	unclassified
					[Eubacterium]	unclassified
						E. biforme
						E. cylindroides
					cc 115	unclassified
					p-75-a5	unclassified

Domain	Phylum	Class	Order	Family	Genus	Species
	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	unclassified
	LD1	unclassified	unclassified	unclassified	unclassified	unclassified
	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	unclassified	unclassified
			Z20	R4-45B	unclassified	unclassified
	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	unclassified	unclassified
	Proteobacteria	Alphaproteobacteria		unclassified	unclassified	unclassified
			RF32	unclassified	unclassified	unclassified
			Rhizobiales	Rhizobiaceae	*Agrobacterium*	unclassified
			Rickettsiales	unclassified	unclassified	unclassified
		Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*	unclassified
				Oxalobacteraceae	*Cupriavidus*	unclassified
					Oxalobacter	unclassified
						O. formigenes
			Neisseriales	Neisseriaceae	*Neisseria*	unclassified
			Tremblayales	unclassified	unclassified	unclassified
		Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	unclassified	unclassified
					Desulfovibrio	unclassified
			GMD14H09	unclassified	unclassified	unclassified
		Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*	unclassified
		Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	unclassified	unclassified
					Succinivibrio	unclassified
			Pasteurellales	Pasteurellaceae	*Aggregatibacter*	unclassified
	Spirochaetes	MVP-15	PL-11B10	unclassified	unclassified	unclassified
		Spirochaetes	Sphaerochaetales	Sphaerochaetaceae	unclassified	unclassified
					Sphaerochaeta	unclassified
			Spirochaetales	Spirochaetaceae	*Treponema*	unclassified
	Synergistetes	Synergistia	Synergistales	unclassified	unclassified	unclassified
				Dethiosulfovibrionaceae	unclassified	unclassified
					TG5	unclassified
				Synergistaceae	unclassified	unclassified
					Candidatus Tammella	unclassified
					vadinCA02	unclassified
	TM7	TM7-3	CW040	unclassified	unclassified	unclassified
			EW055	unclassified	unclassified	unclassified
	Tenericutes	Mollicutes	Acholeplasmatales	unclassified	unclassified	unclassified
				Anaeroplasmataceae	unclassified	unclassified
					Anaeroplasma	unclassified

Domain	Phylum	Class	Order	Family	Genus	Species
			Mycoplasmatales	Mycoplasmataceae	unclassified	unclassified
			RF39	unclassified	unclassified	unclassified
		RF3	ML615J-28	unclassified	unclassified	unclassified
	unclassified	unclassified	unclassified	unclassified	unclassified	unclassified
	Verrucomicrobia	unclassified	unclassified	unclassified	unclassified	unclassified
		Opitutae	HA64	unclassified	unclassified	unclassified
		Verruco-5	WCHB1-41	RFP12	unclassified	unclassified
		Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*	unclassified

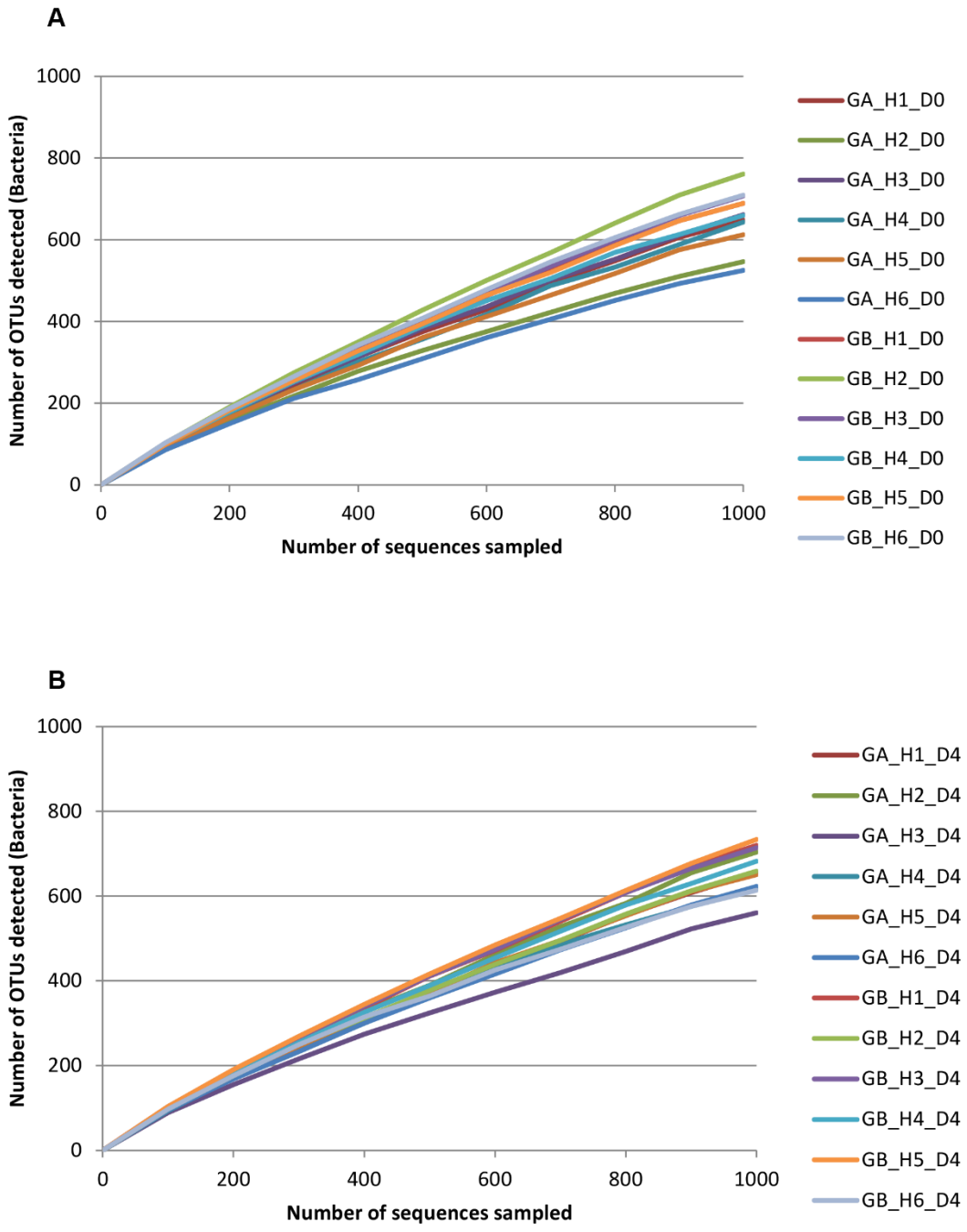
Table S2 B. The faecal archaeal community

Domain	Phylum	Class	Order	Family	Genus	Clade
Archaea	Crenarchaeota	Thermoprotei			*Sulfolobus* and relatives	unclassified
	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	unclassified
					Methanobrevibacter	*Methanobrevibacter arboriphilus* and relatives
						Methanobrevibacter gottschalkii and relatives
						Methanobrevibacter ruminantium and relatives
					Methanosphaera	unclassified
		Methanomicrobia	Methanomicrobiales		*Methanocorpusculum* and relatives	unclassified
			Methanosarcinales	Methanosarcinaceae	*Methanimicrococcus*	unclassified
					Methanosarcina and relatives	unclassified
		Thermoplasmata		Thermoplasmatales	Rumen Cluster C and relatives	unclassified

Table S2 C. The faecal ciliate protozoal community

Domain	Kingdom	Super-phylum	Phylum	Class	Sub-class	Genus
Eukarya	Chromalveolata	Alveolata	Ciliophora	Litostomatea	Haptoria	*Didinium*
						Epiphyllum
						Pelagodileptus
					Trichostomatia	*Blepharocorys*
						Bundleia
						Cochliatoxum
						Cycloposthium
						Diplodinium
						Entodinium
						Eremoplastron
						Isotricha
						Polydiniella
						Spirodinium
						Triadinium
			unclassified		unclassified	unclassified

C-4. Figure S1. Rarefaction curves for bacterial communities in the faeces of individual horses in Groups A and B at two time-points.



Legend: The rarefaction curves show the number of observed species against the depth of sequencing of bacterial communities in the faecal samples from individual horses in Groups A and B on Day 0 (panel A) and Day 4 (panel B). The minimum depth of sequencing per sample for the bacterial group was 1000 sequence reads per sample.

C-5. Table S3. Comparison of the relative abundances of bacterial taxa (genus-level) in the faeces of Group A and B horses on Days 0 and 4 of the study.

Table S3 A) Bacterial genera in the faeces of Group A (fed Diet F) and B horses (fed Diet P) on Day 0 and Day 4 (both groups fed Diet P).

Taxonomic rank within the domain Bacteria Phylum > Class > Order > Family > Genus	Relative abundances on Day 0					Relative abundances on Day 4				
	Group A		Group B			Group A		Group B		
	Median	IQR[a]	Median	IQR[a]	P value[b]	Median	IQR[a]	Median	IQR[a]	P value[b]
Actinobacteria > Coriobacteria > Coriobacteriales > Coriobacteriaceae > unclassified	0.001	0.001 - 0.002	0.005	0.002 - 0.015	0.034	0.005	0.004 - 0.007	0.006	0.004 - 0.010	0.623
Armatimonadetes > SJA-176 > RB046 > unclassified > unclassified	0.001	0.000 - 0.001	0.002	0.001 - 0.003	0.134	0.009	0.006 - 0.017	0.002	0.001 - 0.002	0.011
Bacteroidetes > Bacteroidia > Bacteroidales >unclassified > unclassified	0.079	0.053 - 0.100	0.091	0.080 - 0.132	0.688	0.158	0.132 - 0.177	0.134	0.117 - 0.181	0.574
Bacteroidetes > Bacteroidia > Bacteroidales > BS11 > unclassified	0.006	0.001 - 0.012	0.004	0.002 - 0.007	0.871	0.003	0.001 - 0.006	0.007	0.004 - 0.012	0.260
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > BF311	0.001	0.000 - 0.001	0.010	0.009 - 0.014	0.003*	0.007	0.001 - 0.021	0.011	0.008 - 0.011	0.935
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > *Bacteroides*	0.001	0.000 - 0.005	0.001	0.000 - 0.002	1.000	0.002	0.001 - 0.007	0.003	0.002 - 0.003	0.869
Bacteroidetes > Bacteroidia > Bacteroidales > Porphyromonadaceae > *Paludibacter*	0.000	0.000 - 0.001	0.001	0.000 - 0.001	0.476	0.000	0.000 - 0.000	0.001	0.000 - 0.001	0.390
Bacteroidetes > Bacteroidia > Bacteroidales > Porphyromonadaceae > *Parabacteroides*	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.317	0.004	0.000 - 0.024	0.000	0.000 - 0.000	0.058
Bacteroidetes > Bacteroidia > Bacteroidales > Prevotellaceae > unclassified	0.000	0.000 - 0.003	0.001	0.001 - 0.002	0.402	0.001	0.001 - 0.002	0.000	0.000 - 0.001	0.014
Bacteroidetes > Bacteroidia > Bacteroidales > Prevotellaceae > *Prevotella*	0.010	0.003 - 0.024	0.027	0.020 - 0.039	0.172	0.028	0.020 - 0.065	0.035	0.026 - 0.048	1.000
Bacteroidetes > Bacteroidia > Bacteroidales > RF16 > unclassified	0.002	0.000 - 0.003	0.002	0.000 - 0.002	0.741	0.002	0.001 - 0.004	0.001	0.000 - 0.002	0.138
Bacteroidetes > Bacteroidia > Bacteroidales > S24-7 > unclassified	0.007	0.004 - 0.011	0.003	0.002 - 0.003	0.050	0.002	0.001 - 0.005	0.003	0.003 - 0.006	0.410
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > unclassified	0.006	0.002 - 0.012	0.010	0.007 - 0.015	0.261	0.007	0.005 - 0.015	0.012	0.011 - 0.016	0.148
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > CF231	0.002	0.000 - 0.003	0.012	0.008 - 0.022	0.004*	0.004	0.002 - 0.011	0.011	0.006 - 0.014	0.091
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.040	0.006 - 0.063	0.043	0.037 - 0.067	0.470	0.023	0.017 - 0.025	0.031	0.022 - 0.050	0.336
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > [Prevotella]	0.005	0.002 - 0.010	0.006	0.001 - 0.010	0.872	0.003	0.001 - 0.005	0.003	0.001 - 0.008	0.806
Cyanobacteria > 4C0d-2 > YS2 > unclassified > unclassified	0.004	0.001 - 0.004	0.001	0.000 - 0.002	0.121	0.002	0.000 - 0.002	0.002	0.001 - 0.007	0.404
Fibrobacteres > Fibrobacteria > Fibrobacterales > Fibrobacteraceae > *Fibrobacter*	0.003	0.002 - 0.005	0.005	0.003 - 0.009	0.197	0.008	0.004 - 0.010	0.010	0.008 - 0.017	0.199
Firmicutes > Clostridia > unclassified > unclassified > unclassified	0.001	0.000 - 0.003	0.000	0.000 - 0.001	0.281	0.002	0.000 - 0.003	0.002	0.001 - 0.002	0.867
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.112	0.103 - 0.131	0.163	0.149 - 0.181	0.003*	0.151	0.130 - 0.167	0.146	0.131 - 0.164	0.872
Firmicutes > Clostridia > Clostridiales > Christensenellaceae > unclassified	0.007	0.004 - 0.010	0.005	0.004 - 0.006	0.462	0.004	0.002 - 0.007	0.006	0.003 - 0.007	0.685
Firmicutes > Clostridia > Clostridiales > Clostridiaceae > unclassified	0.006	0.003 - 0.017	0.006	0.004 - 0.008	0.872	0.005	0.004 - 0.005	0.006	0.003 - 0.012	0.808
Firmicutes > Clostridia > Clostridiales > Clostridiaceae > *Clostridium*	0.010	0.007 - 0.015	0.010	0.006 - 0.014	0.747	0.006	0.005 - 0.008	0.013	0.007 - 0.015	0.335
Firmicutes > Clostridia > Clostridiales > Eubacteriaceae > *Pseudoramibacter_Eubacterium*	0.001	0.000 - 0.002	0.002	0.000 - 0.004	0.359	0.006	0.004 - 0.007	0.003	0.002 - 0.005	0.166
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.098	0.067 - 0.139	0.188	0.157 - 0.238	0.006*	0.173	0.151 - 0.195	0.179	0.167 - 0.208	0.521
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > *Blautia*	0.006	0.003 - 0.008	0.012	0.007 - 0.015	0.172	0.004	0.002 - 0.009	0.007	0.005 - 0.009	0.374
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > *Coprococcus*	0.005	0.005 - 0.007	0.011	0.008 - 0.011	0.062	0.017	0.011 - 0.021	0.011	0.008 - 0.012	0.062
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.395	0.387 - 0.413	0.281	0.227 - 0.293	0.016	0.205	0.171 - 0.220	0.226	0.158 - 0.290	0.748
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Oscillospira*	0.015	0.013 - 0.019	0.008	0.006 - 0.008	0.044	0.009	0.003 - 0.010	0.006	0.004 - 0.011	0.936

Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.045	0.028 - 0.058	0.019	0.015 - 0.030	0.054	0.025	0.018 - 0.032	0.024	0.017 - 0.037	1.000
Firmicutes > Clostridia > Clostridiales > [Mogibacteriaceae] > unclassified	0.009	0.008 - 0.011	0.011	0.007 - 0.012	0.872	0.017	0.012 - 0.033	0.015	0.012 - 0.016	0.331
Firmicutes > Clostridia > Clostridiales > [Mogibacteriaceae] > *Mogibacterium*	0.006	0.001 - 0.013	0.005	0.004 - 0.005	0.416	0.006	0.005 - 0.008	0.005	0.002 - 0.009	0.420
Firmicutes > Erysipelotrichi > Erysipelotrichales > Erysipelotrichaceae > RFN20	0.008	0.003 - 0.013	0.003	0.003 - 0.008	0.135	0.010	0.008 - 0.015	0.002	0.002 - 0.004	0.004*
Firmicutes > Erysipelotrichi > Erysipelotrichales > Erysipelotrichaceae > p-75-a5	0.004	0.000 - 0.006	0.002	0.001 - 0.002	0.329	0.004	0.002 - 0.008	0.001	0.000 - 0.002	0.059
Planctomycetes > Planctomycetia > Pirellulales > Pirellulaceae > unclassified	0.001	0.000 - 0.003	0.000	0.000 - 0.000	0.181	0.001	0.000 - 0.002	0.001	0.000 - 0.001	0.445
Proteobacteria > Alphaproteobacteria > RF32 > unclassified > unclassified	0.000	0.000 - 0.001	0.000	0.000 - 0.001	1.000	0.001	0.000 - 0.001	0.000	0.000 - 0.001	0.575
Proteobacteria > Betaproteobacteria > Tremblayales > unclassified > unclassified	0.000	0.000 - 0.000	0.000	0.000 - 0.001	0.138	0.000	0.000 - 0.000	0.001	0.000 - 0.002	0.209
Proteobacteria > Gammaproteobacteria > Aeromonadales > Succinivibrionaceae > unclassified	0.000	0.000 - 0.002	0.000	0.000 - 0.000	0.139	0.000	0.000 - 0.000	0.000	0.000 - 0.000	n/p
Spirochaetes > Spirochaetes > Spirochaetales > Spirochaetaceae > *Treponema*	0.005	0.004 - 0.006	0.002	0.002 - 0.004	0.101	0.002	0.001 - 0.005	0.005	0.003 - 0.007	0.078
Synergistetes > Synergistia > Synergistales > Synergistaceae > unclassified	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.317	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.902
Other Genera <1%	0.028	0.022 - 0.029	0.033	0.029 - 0.036	0.077	0.030	0.029 - 0.032	0.032	0.026 - 0.038	0.518

[a] IQR – Interquartile range

[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$

*Differences between Groups A and B

n/p – no P value

Table S3 B) Bacterial genera in the faeces of Group A horses on Day 0 (fed Diet F) and Day 4 (fed Diet P).

Taxonomic rank within the domain Bacteria **Phylum > Class > Order > Family > Genus**	**Relative abundances of Group A horses**				
	Day 0		**Day 4**		
	Median	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**
Actinobacteria > Coriobacteria > Coriobacteriales > Coriobacteriaceae > unclassified	0.001	0.001 - 0.002	0.005	0.004 - 0.007	0.050
Armatimonadetes > SJA-176 > RB046 > unclassified > unclassified	0.001	0.000 - 0.001	0.009	0.006 - 0.017	0.005*
Bacteroidetes > Bacteroidia > Bacteroidales >unclassified > unclassified	0.079	0.053 - 0.100	0.158	0.132 - 0.177	0.109
Bacteroidetes > Bacteroidia > Bacteroidales > BS11 > unclassified	0.006	0.001 - 0.012	0.003	0.001 - 0.006	0.809
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > BF311	0.001	0.000 - 0.001	0.007	0.001 - 0.021	0.085
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > *Bacteroides*	0.001	0.000 - 0.005	0.002	0.001 - 0.007	0.413
Bacteroidetes > Bacteroidia > Bacteroidales > Porphyromonadaceae > *Paludibacter*	0.000	0.000 - 0.001	0.000	0.000 - 0.000	0.598
Bacteroidetes > Bacteroidia > Bacteroidales > Porphyromonadaceae > *Parabacteroides*	0.000	0.000 - 0.000	0.004	0.000 - 0.024	0.153
Bacteroidetes > Bacteroidia > Bacteroidales > Prevotellaceae > unclassified	0.000	0.000 - 0.003	0.001	0.001 - 0.002	0.212
Bacteroidetes > Bacteroidia > Bacteroidales > Prevotellaceae > *Prevotella*	0.010	0.003 - 0.024	0.028	0.020 - 0.065	0.078
Bacteroidetes > Bacteroidia > Bacteroidales > RF16 > unclassified	0.002	0.000 - 0.003	0.002	0.001 - 0.004	0.370
Bacteroidetes > Bacteroidia > Bacteroidales > S24-7 > unclassified	0.007	0.004 - 0.011	0.002	0.001 - 0.005	0.106
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > unclassified	0.006	0.002 - 0.012	0.007	0.005 - 0.015	0.519
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > CF231	0.002	0.000 - 0.003	0.004	0.002 - 0.011	0.169
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.040	0.006 - 0.063	0.023	0.017 - 0.025	0.749
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > [Prevotella]	0.005	0.002 - 0.010	0.003	0.001 - 0.005	0.332
Cyanobacteria > 4C0d-2 > YS2 > unclassified > unclassified	0.004	0.001 - 0.004	0.002	0.000 - 0.002	0.166
Fibrobacteres > Fibrobacteria > Fibrobacterales > Fibrobacteraceae > *Fibrobacter*	0.003	0.002 - 0.005	0.008	0.004 - 0.010	0.076
Firmicutes > Clostridia > unclassified > unclassified > unclassified	0.001	0.000 - 0.003	0.002	0.000 - 0.003	0.613
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.112	0.103 - 0.131	0.151	0.130 - 0.167	0.078
Firmicutes > Clostridia > Clostridiales > Christensenellaceae > unclassified	0.007	0.004 - 0.010	0.004	0.002 - 0.007	0.318
Firmicutes > Clostridia > Clostridiales > Clostridiaceae > unclassified	0.006	0.003 - 0.017	0.005	0.004 - 0.005	0.571
Firmicutes > Clostridia > Clostridiales > Clostridiaceae > *Clostridium*	0.010	0.007 - 0.015	0.006	0.005 - 0.008	0.092
Firmicutes > Clostridia > Clostridiales > Eubacteriaceae > *Pseudoramibacter_Eubacterium*	0.001	0.000 - 0.002	0.006	0.004 - 0.007	0.004*
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.098	0.067 - 0.139	0.173	0.151 - 0.195	0.025
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > *Blautia*	0.006	0.003 - 0.008	0.004	0.002 - 0.009	0.630
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > *Coprococcus*	0.005	0.005 - 0.007	0.017	0.011 - 0.021	0.024
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.395	0.387 - 0.413	0.205	0.171 - 0.220	0.004*
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Oscillospira*	0.015	0.013 - 0.019	0.009	0.003 - 0.010	0.025
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.045	0.028 - 0.058	0.025	0.018 - 0.032	0.146
Firmicutes > Clostridia > Clostridiales > [Mogibacteriaceae] > unclassified	0.009	0.008 - 0.011	0.017	0.012 - 0.033	0.024
Firmicutes > Clostridia > Clostridiales > [Mogibacteriaceae] > *Mogibacterium*	0.006	0.001 - 0.013	0.006	0.005 - 0.008	0.747
Firmicutes > Erysipelotrichi > Erysipelotrichales > Erysipelotrichaceae > RFN20	0.008	0.003 - 0.013	0.010	0.008 - 0.015	0.376
Firmicutes > Erysipelotrichi > Erysipelotrichales > Erysipelotrichaceae > p-75-a5	0.004	0.000 - 0.006	0.004	0.002 - 0.008	0.746
Planctomycetes > Planctomycetia > Pirellulales > Pirellulaceae > unclassified	0.001	0.000 - 0.003	0.001	0.000 - 0.002	0.867
Proteobacteria > Alphaproteobacteria > RF32 > unclassified > unclassified	0.000	0.000 - 0.001	0.001	0.000 - 0.001	0.784
Proteobacteria > Betaproteobacteria > Tremblayales > unclassified > unclassified	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.314
Proteobacteria > Gammaproteobacteria > Aeromonadales > Succinivibrionaceae > unclassified	0.000	0.000 - 0.002	0.000	0.000 - 0.000	0.140

Spirochaetes > Spirochaetes > Spirochaetales > Spirochaetaceae > *Treponema*	0.005	0.004 - 0.006	0.002	0.001 - 0.005	0.060
Synergistetes > Synergistia > Synergistales > Synergistaceae > unclassified	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.902
Other Genera <1%	0.028	0.022 - 0.029	0.030	0.029 - 0.032	0.170

[a] IQR – Interquartile range

[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$

*Differences between Days 0 and 4

Table S3 C) Bacterial genera in the faeces of Group B horses on Days 0 and 4 (fed Diet P).

Taxonomic rank within the domain Bacteria **Phylum > Class > Order > Family > Genus**	**Relative abundances of Group B horses**				
	Day 0		**Day 4**		
	Median	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**
Actinobacteria > Coriobacteria > Coriobacteriales > Coriobacteriaceae > unclassified	0.005	0.002 - 0.015	0.006	0.004 - 0.010	0.872
Armatimonadetes > SJA-176 > RB046 > unclassified > unclassified	0.002	0.001 - 0.003	0.002	0.001 - 0.002	0.868
Bacteroidetes > Bacteroidia > Bacteroidales >unclassified > unclassified	0.091	0.080 - 0.132	0.134	0.117 - 0.181	0.128
Bacteroidetes > Bacteroidia > Bacteroidales > BS11 > unclassified	0.004	0.002 - 0.007	0.007	0.004 - 0.012	0.290
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > BF311	0.010	0.009 - 0.014	0.011	0.008 - 0.011	0.566
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > *Bacteroides*	0.001	0.000 - 0.002	0.003	0.002 - 0.003	0.070
Bacteroidetes > Bacteroidia > Bacteroidales > Porphyromonadaceae > *Paludibacter*	0.001	0.000 - 0.001	0.001	0.000 - 0.001	0.859
Bacteroidetes > Bacteroidia > Bacteroidales > Porphyromonadaceae > *Parabacteroides*	0.000	0.000 - 0.000	0.000	0.000 - 0.000	n/p
Bacteroidetes > Bacteroidia > Bacteroidales > Prevotellaceae > unclassified	0.001	0.001 - 0.002	0.000	0.000 - 0.001	0.058
Bacteroidetes > Bacteroidia > Bacteroidales > Prevotellaceae > *Prevotella*	0.027	0.020 - 0.039	0.035	0.026 - 0.048	0.470
Bacteroidetes > Bacteroidia > Bacteroidales > RF16 > unclassified	0.002	0.000 - 0.002	0.001	0.000 - 0.002	0.804
Bacteroidetes > Bacteroidia > Bacteroidales > S24-7 > unclassified	0.003	0.002 - 0.003	0.003	0.003 - 0.006	0.347
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > unclassified	0.010	0.007 - 0.015	0.012	0.011 - 0.016	0.294
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > CF231	0.012	0.008 - 0.022	0.011	0.006 - 0.014	0.421
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.043	0.037 - 0.067	0.031	0.022 - 0.050	0.173
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > [Prevotella]	0.006	0.001 - 0.010	0.003	0.001 - 0.008	0.685
Cyanobacteria > 4C0d-2 > YS2 > unclassified > unclassified	0.001	0.000 - 0.002	0.002	0.001 - 0.007	0.139
Fibrobacteres > Fibrobacteria > Fibrobacterales > Fibrobacteraceae > *Fibrobacter*	0.005	0.003 - 0.009	0.010	0.008 - 0.017	0.092
Firmicutes > Clostridia > unclassified > unclassified > unclassified	0.000	0.000 - 0.001	0.002	0.001 - 0.002	0.028
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.163	0.149 - 0.181	0.146	0.131 - 0.164	0.128
Firmicutes > Clostridia > Clostridiales > Christensenellaceae > unclassified	0.005	0.004 - 0.006	0.006	0.003 - 0.007	0.746
Firmicutes > Clostridia > Clostridiales > Clostridiaceae > unclassified	0.006	0.004 - 0.008	0.006	0.003 - 0.012	0.809
Firmicutes > Clostridia > Clostridiales > Clostridiaceae > *Clostridium*	0.010	0.006 - 0.014	0.013	0.007 - 0.015	0.687
Firmicutes > Clostridia > Clostridiales > Eubacteriaceae > *Pseudoramibacter_Eubacterium*	0.002	0.000 - 0.004	0.003	0.002 - 0.005	0.464
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.188	0.157 - 0.238	0.179	0.167 - 0.208	0.810
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > *Blautia*	0.012	0.007 - 0.015	0.007	0.005 - 0.009	0.090
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > *Coprococcus*	0.011	0.008 - 0.011	0.011	0.008 - 0.012	0.684
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.281	0.227 - 0.293	0.226	0.158 - 0.290	0.378
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Oscillospira*	0.008	0.006 - 0.008	0.006	0.004 - 0.011	0.520
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.019	0.015 - 0.030	0.024	0.017 - 0.037	0.575
Firmicutes > Clostridia > Clostridiales > [Mogibacteriaceae] > unclassified	0.011	0.007 - 0.012	0.015	0.012 - 0.016	0.092
Firmicutes > Clostridia > Clostridiales > [Mogibacteriaceae] > *Mogibacterium*	0.005	0.004 - 0.005	0.005	0.002 - 0.009	0.808
Firmicutes > Erysipelotrichi > Erysipelotrichales > Erysipelotrichaceae > RFN20	0.003	0.003 - 0.008	0.002	0.002 - 0.004	0.290
Firmicutes > Erysipelotrichi > Erysipelotrichales > Erysipelotrichaceae > p-75-a5	0.002	0.001 - 0.002	0.001	0.000 - 0.002	0.316
Planctomycetes > Planctomycetia > Pirellulales > Pirellulaceae > unclassified	0.000	0.000 - 0.000	0.001	0.000 - 0.001	0.211
Proteobacteria > Alphaproteobacteria > RF32 > unclassified > unclassified	0.000	0.000 - 0.001	0.000	0.000 - 0.001	0.847
Proteobacteria > Betaproteobacteria > Tremblayales > unclassified > unclassified	0.000	0.000 - 0.001	0.001	0.000 - 0.002	0.162
Proteobacteria > Gammaproteobacteria > Aeromonadales > Succinivibrionaceae > unclassified	0.000	0.000 - 0.000	0.000	0.000 - 0.000	n/p

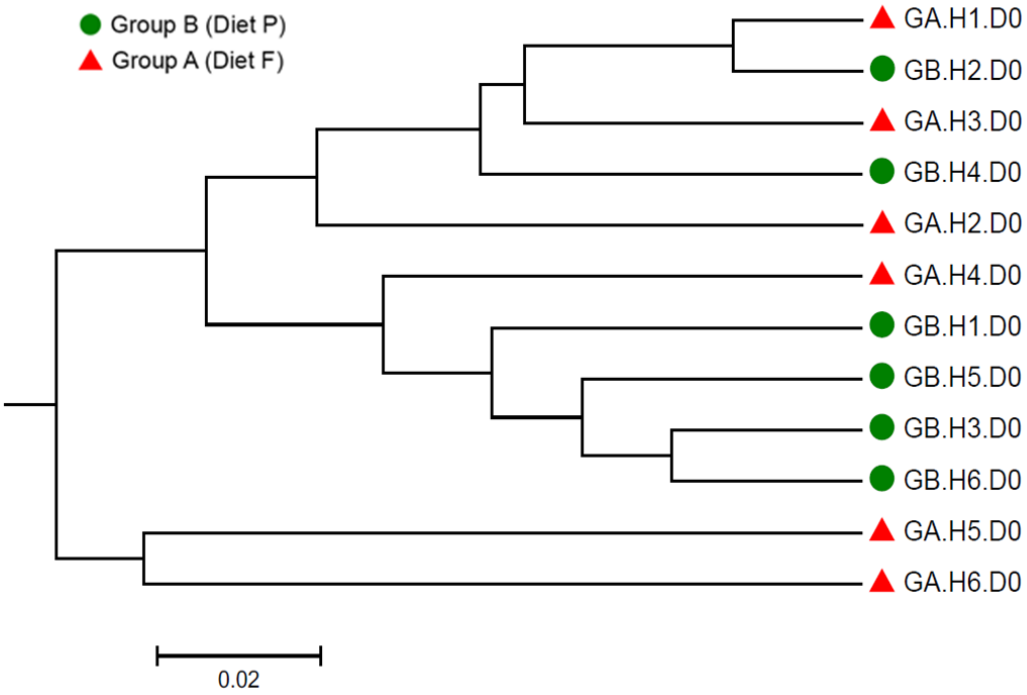
Spirochaetes > Spirochaetes > Spirochaetales > Spirochaetaceae > *Treponema*	0.002	0.002 - 0.004	0.005	0.003 - 0.007	0.075
Synergistetes > Synergistia > Synergistales > Synergistaceae > unclassified	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.317
Other Genera <1%	0.033	0.029 - 0.036	0.032	0.026 - 0.038	0.936

[a] IQR – Interquartile range

[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$

n/p – no P value

C-6. UPGMA dendrograme based on the Sørensen-Dice dissimilarity metric of faecal sample on Day 0.



Legend: The Sørensen-Dice dissimilarity metric takes into account the presence and absence of taxa, to generate a dissimilarity matrix for the faecal microbiota present in samples obtained from horses on Day 0. The UPGMA dendrogram showed no clustering of horses by diet group (H; numbered from 1-6 per group), indicating that the faecal bacterial community structure on Day 0 may not be associated with the presence or absence of specific bacterial taxa in Group A or B horses, which were fed Diet F and P, respectively.

C-7. Diversity indices for bacterial taxa in the faeces of Group B horses.

Diversity Indices	Day 0		Day 4		
	Median	IQR[a]	Median	IQR[a]	P value
Simpson	0.85	0.84 - 0.85	0.86	0.85 - 0.87	0.12
Shannon-Weiner	2.35	2.28 - 2.38	2.43	2.42 - 2.49	0.05
Evenness	0.31	0.31 - 0.32	0.32	0.31 - 0.36	0.20

[a] IQR – Interquartile range

Legend: There were no significant differences in the diversity indices of Group B horses on Days 0 and 4.

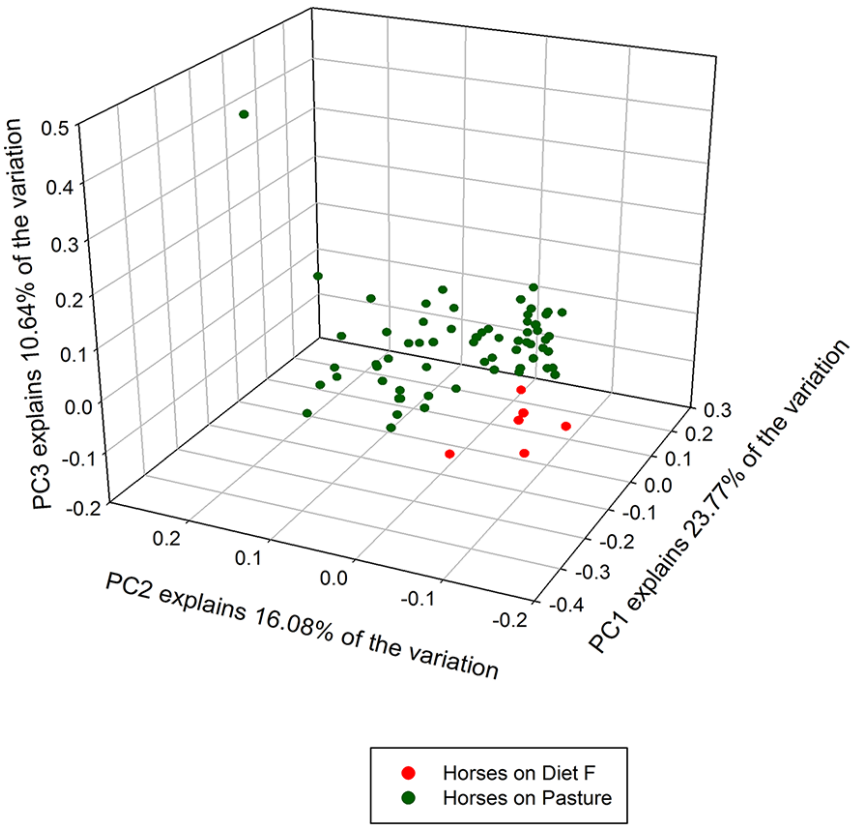
C-8. Comparison of relative abundances of bacterial families in the faeces of Groups A and B horses on Day 4.

	Group A		Group B		
	Median	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**
Ruminococcaceae	0.241	0.199 - 0.271	0.261	0.223 - 0.325	0.52
Lachnospiraceae	0.205	0.172 - 0.249	0.199	0.192 - 0.223	0.87
Unclassified family within order Bacteroidales	0.158	0.132 - 0.177	0.134	0.117 - 0.181	0.57
Unclassified family within order Clostridiales	0.151	0.130 - 0.167	0.146	0.131 - 0.164	0.87
[Paraprevotellaceae]	0.039	0.026 - 0.059	0.054	0.049 - 0.084	0.26
Prevotellaceae	0.030	0.021 - 0.072	0.036	0.026 - 0.048	1.00
[Mogibacteriaceae]	0.027	0.020 - 0.040	0.021	0.017 - 0.021	0.14
Erysipelotrichaceae	0.019	0.013 - 0.026	0.007	0.006 - 0.009	0.01
Bacteroidaceae	0.018	0.009 - 0.027	0.015	0.012 - 0.016	0.74
Clostridiaceae	0.011	0.008 - 0.013	0.020	0.016 - 0.029	0.22
Unclassified family inder order RB046	0.009	0.006 - 0.017	0.002	0.001 - 0.002	0.01
Fibrobacteraceae	0.008	0.004 - 0.010	0.010	0.008 - 0.017	0.20
Coriobacteriaceae	0.007	0.006 - 0.009	0.008	0.007 - 0.011	0.37
Eubacteriaceae	0.007	0.005 - 0.007	0.004	0.002 - 0.005	0.14
Veillonellaceae	0.007	0.005 - 0.007	0.004	0.003 - 0.005	0.09
Porphyromonadaceae	0.006	0.000 - 0.024	0.001	0.000 - 0.001	0.14
Christensenellaceae	0.004	0.002 - 0.007	0.006	0.003 - 0.007	0.68
BS11	0.003	0.001 - 0.006	0.007	0.004 - 0.012	0.26
RF16	0.002	0.001 - 0.004	0.001	0.000 - 0.002	0.14
S24-7	0.002	0.001 - 0.005	0.003	0.003 - 0.006	0.42
Unclassified family within order YS2	0.002	0.000 - 0.002	0.002	0.001 - 0.007	0.42
Unclassified family within class Clostridia	0.002	0.000 - 0.003	0.002	0.001 - 0.002	0.87
Spirochaetaceae	0.002	0.001 - 0.005	0.005	0.003 - 0.007	0.07
Pirellulaceae	0.001	0.000 - 0.002	0.001	0.000 - 0.001	0.47
Unclassified family within order RF32	0.001	0.000 - 0.001	0.000	0.000 - 0.001	0.63
Synergistaceae	0.001	0.000 - 0.001	0.000	0.000 - 0.001	0.63
Unclassified family within order Tremblayales	0.000	0.000 - 0.000	0.001	0.000 - 0.002	0.26
Succinivibrionaceae	0.000	0.000 - 0.000	0.000	0.000 - 0.000	NA
Other families <1%	0.009	0.007 - 0.012	0.010	0.009 - 0.011	0.29

[a]IQR – Interquartile range
[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$

Legend: There were no significant differences between the relative abundances of bacterial families detected in the faeces of Groups A and B horses on Day 4.

C-9. Figure S4. 3-dimensional plot of bacterial communities in all 72 samples.



Legend: The graph contains the data of the bacterial communities in the faeces of horses in Groups A and B at six time-points over the 3-week period. The graph indicates that the horses fed pasture (Diet P, green), from both Groups A (Days 4-21) and B (Days 0-21), clustered separately from the horses fed ensiled conserved forage-grain (Diet F, red) on Day 0.

C-10. Diversity indices for archaeal taxa in the faeces of Group A and B horses.

A. Comaprison between treatment days.

	Day 0					Day 4				
Diversity	**Group A**		**Group B**			**Group A**		**Group B**		
Indices	**Median**	**IQR[a]**	**Median**	**IQR[a]**	**P value**	**Median**	**IQR[a]**	**Median**	**IQR[a]**	**P value**
Simpson	0.48	0.19 - 0.55	0.42	0.36 - 0.46	0.46	0.42	0.40 - 0.48	0.45	0.41 - 0.49	0.42
Shannon-Weiner	0.81	0.44 - 0.95	0.68	0.60 - 0.72	0.36	0.70	0.67 - 0.70	0.70	0.68 - 0.75	0.63
Evenness	0.50	0.31 - 0.61	0.51	0.45 - 0.68	0.46	0.58	0.44 - 0.67	0.54	0.49 - 0.68	0.87

[a] IQR – Interquartile range

Legend: There were no significant differences in the diversity indices of the faecal archaeal community between Group A and B horses on either Day 0 or Day 4.

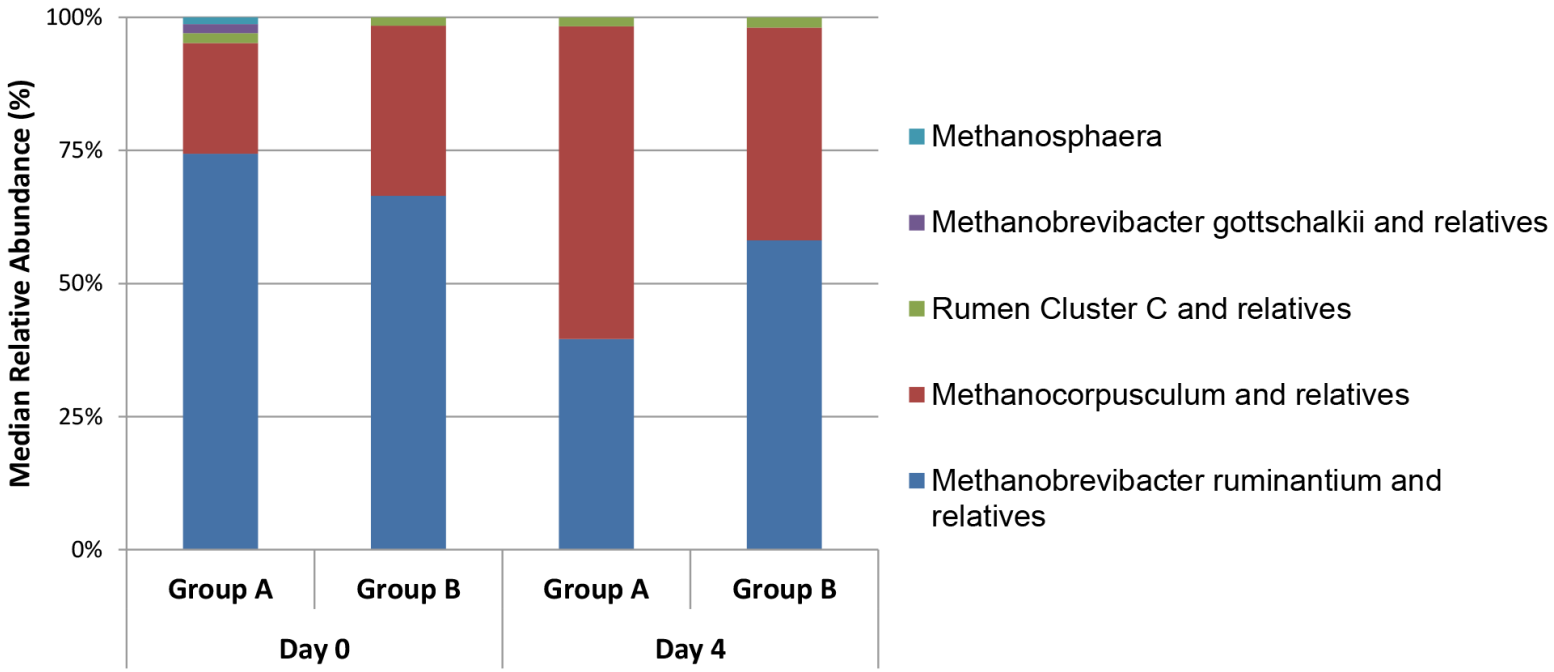
B. Comparison between treatment groups.

	Group A					Group B				
Diversity	**Day 0**		**Day 4**			**Day 0**		**Day 4**		
Indices	**Median**	**IQR[a]**	**Median**	**IQR[a]**	**P value**	**Median**	**IQR[a]**	**Median**	**IQR[a]**	**P value**
Simpson	0.48	0.19 - 0.55	0.42	0.40 - 0.48	0.42	0.42	0.36 - 0.46	0.45	0.41 - 0.49	0.31
Shannon-Weiner	0.81	0.44 - 0.95	0.70	0.67 - 0.70	0.26	0.68	0.60 - 0.72	0.70	0.68 - 0.75	0.31
Evenness	0.50	0.31 - 0.61	0.58	0.44 - 0.67	0.26	0.51	0.45 - 0.68	0.54	0.49 - 0.68	0.93

[a] IQR – Interquartile range

Legend: There were no significant differences in the diversity indices of the faecal archaeal community between Group A horses on Days 0 and 4 and between Group B horses on Days 0 and 4.

C-11. Figure S2. Relative abundance of archaeal clades in the faecal microbial community of horses.



Legend: The chart shows the median relative abundance of archaeal clades in the faeces of horses in Groups A and B, and indicates the dominance of two clades; *Methanocorpusculum* and relatives and *Methanobrevibacter ruminantium* and relatives. The colours in the figure legend show the archaeal clades with median relative abundances > 15%.

C-12. Table S4. Comparison of the relative abundances of archaeal taxa (clade-level) in the faeces of Group A and B horses on Days 0 and 4 of the study.

Table S4 A) Group A horses fed two different diets in Day 0 (Diet F) and Day 4 (Diet P)

Taxonomic rank under the domain Archaea	**Relative abundances of Group A horses**				
Phylum > Class > Order > Family > Genus / Clade	**Day 0**		**Day 4**		
	Median	**IQR[a]**	**Median**	**IQR**	**P value[b]**
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_ruminantium*_and_relatives	0.630	0.413 - 0.897	0.398	0.244 - 0.719	0.200
Euryarchaeota > Methanomicrobia > Methanomicrobiales > *Genera_incertae_sedis* > *Methanocorpusculum*_and_relatives	0.177	0.056 - 0.309	0.591	0.253 - 0.722	0.109
Euryarchaeota > Thermoplasmata > Thermoplasmatales > Rumen Cluster C_and_relatives	0.016	0.003 - 0.031	0.017	0.009 - 0.034	0.809
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_gottschalkii*_and_relatives	0.014	0.000 - 0.028	0.000	0.000 - 0.000	0.060
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanosphaera*	0.011	0.003 - 0.025	0.000	0.000 - 0.000	0.016
Other Taxa <1%	0.000	0.000 - 0.000	0.000	0.000 - 0.000	1.000

[a] IQR – Interquartile range
[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.008$

Table S4 B) Group B horses fed pasture (Diet P) on Days 0 and 4

Taxonomic rank under the domain Archaea	**Relative abundances of Group B horses**				
Phylum > Class > Order > Family > Genus / Clade	**Day 0**		**Day 4**		
	Median	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_ruminantium*_and_relatives	0.663	0.569 - 0.769	0.575	0.431 - 0.722	0.465
Euryarchaeota > Methanomicrobia > Methanomicrobiales > *Genera_incertae_sedis* > *Methanocorpusculum*_and_relatives	0.319	0.219 - 0.431	0.395	0.253 - 0.569	0.465
Euryarchaeota > Thermoplasmata > Thermoplasmatales > Rumen Cluster C_and_relatives	0.016	0.009 - 0.038	0.019	0.016 - 0.034	1.000
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_gottschalkii*_and_relatives	0.000	0.000 - 0.003	0.000	0.000 - 0.003	0.827
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanosphaera*	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.273
Other Taxa <1%	0.000	0.000 - 0.000	0.002	0.000 - 0.003	0.081

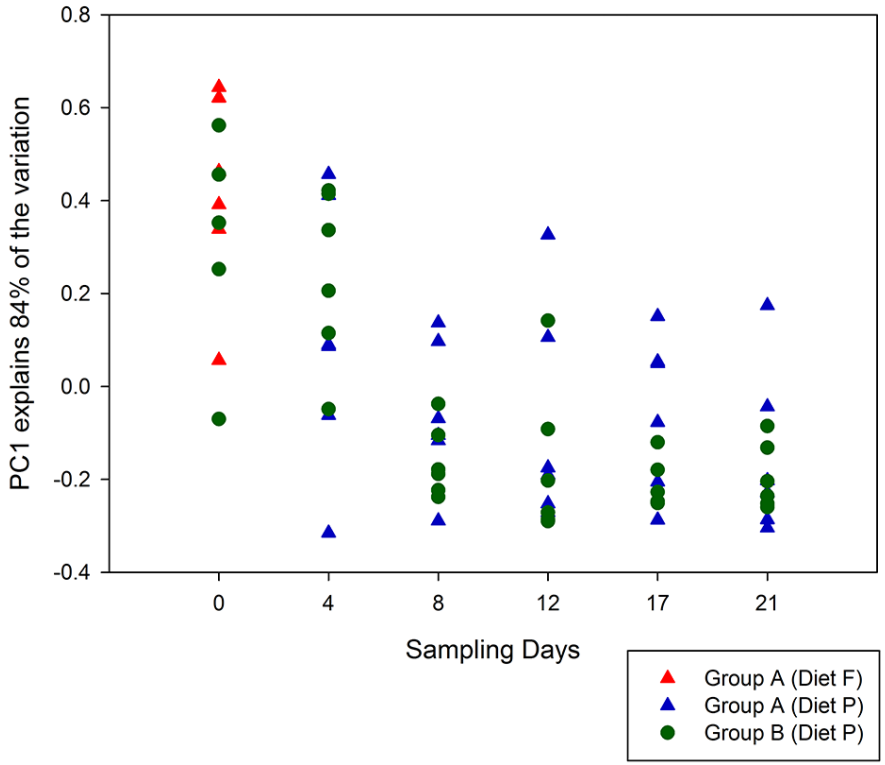
[a] IQR – Interquartile range
[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.008$

Table S4 C) Group A and B horses fed pasture (Diet P) on Day 4

Taxonomic rank under the domain Archaea	**Relative abundances on Day 4**				
Phylum > Class > Order > Family > Genus / Clade	**Group A**		**Group B**		
	Median	**IQR[a]**	**Median**	**IQR[a]**	**P value[b]**
Euryarchaeota > Methanomicrobia > Methanomicrobiales > *Genera_incertae_sedis* > *Methanocorpusculum*_and_relatives	0.591	0.253 - 0.722	0.395	0.253 - 0.569	0.370
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_ruminantium*_and_relatives	0.398	0.244 - 0.719	0.575	0.431 - 0.722	0.330
Euryarchaeota > Thermoplasmata > Thermoplasmatales > Rumen Cluster C_and_relatives	0.017	0.009 - 0.034	0.019	0.016 - 0.034	0.930
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_gottschalkii*_and_relatives	0.000	0.000 - 0.000	0.000	0.000 - 0.003	0.670
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanosphaera*	0.000	0.000 - 0.000	0.000	0.000 - 0.000	0.310
Other Taxa <1%	0.000	0.000 - 0.000	0.002	0.000 - 0.003	0.210

[a] IQR – Interquartile range
[b] Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.008$

C-13. Figure S3. Principal coordinate plot for data on the archaeal community structure in the faeces of horses across all sampling days based on Bray-Curtis dissimilarity.



Legend: The plot illustrates the similarities in the faecal archaeal communities in the faeces of horses in Groups A and B on six sampling time-points over a period of three weeks. Clustering of horses by diet was not observed on Day 0, and no clustering was seen when horses were fed pasture (Diet P) from Days 4-21, with 84% of the variation explained by PC1.

CHAPTER 6

SEASONAL CHANGES IN PASTURE AND FAECAL MICROBIOTA OF HORSES

(Horses on Pasture Trial)

PRELUDE TO CHAPTER 6

In New Zealand, many horses that were kept on pasture all year round maintained their body weight and a higher than normal body condition, despite potential fluctuations in the availability of pasture cover across seasons (Chapters 3 and 4). However, little is known about the stability of the faecal microbiota of horses kept on pasture and its association with seasonal changes in pasture composition. The results of Chapter 5 showed that the faecal microbiota profile of Thoroughbred horses was diet-specific, but the diversity of the faecal bacterial community appeared to fluctuate during the 3-week study period when the horses were grazing on typical New Zealand pasture. It was hypothesised that these fluctuations in bacterial diversity may be associated with changes in the pasture composition, and this required further investigation.

The experiment in Chapter 6 utilised a more advanced sequencing technique (Illumina MiSeq) to examine the seasonal variation in the faecal microbiota of horses on pasture, and correlate the changes observed in microbial populations to pasture composition and climatic factors. Chapter 6 reports on the diversity and community structure of faecal bacteria assessed over a 12-month period, in a cohort of horses kept on pasture and supplemented with hay during the winter months.

Supplementary information on the methodology and results of data analysis are presented in the appendix for this chapter (Appendix D). Chapter 6 is based on a manuscript submitted to the Journal Public Library of Science One (PLoS ONE).

Fernandes, K.A., Gee, E.K., Rogers, C.W., Kittelmann, S., Biggs, P.J., Bermingham, E.N., Bolwell, C.F. and Thomas, D.G. (Submitted 03.07.2016). Seasonal variation in the faecal microbiota of mature adult horses maintained on pasture in New Zealand. *PLoS ONE*.

CHAPTER 6: SEASONAL VARIATION IN THE FAECAL MICROBIOTA OF MATURE ADULT HORSES MAINTAINED ON PASTURE IN NEW ZEALAND

6.1 Abstract

Seasonal variation in the faecal microbiota of forage-fed horses was investigated over a 12-month period to determine whether the bacterial diversity fluctuated over time. Horses (n=10) were maintained on pasture for one year, with hay supplemented from June to October. At monthly intervals, data were recorded on pasture availability and climate (collected continuously and averaged on monthly basis), pasture and hay samples were collected for nutrient analysis, and faecal samples were collected from all horses to investigate the diversity of faecal microbiota using next-generation sequencing on the Illumina MiSeq platform. The alpha diversity of bacterial genera was high in all samples (n=118), with significantly higher Simpson's ($P<0.001$) and Shannon-Wiener ($P<0.001$) diversity indices observed during the months when horses were kept exclusively on pasture compared to the months when pasture was supplemented with hay. There were significant effects of diet, season and month (ANOSIM, $P<0.01$ for each comparison) on the beta diversity of bacterial genera identified in the faeces. While there was some inter-horse variation, hierarchical clustering of beta diversity indices showed separate clades originating for samples obtained during May, June and July (late-autumn to winter period), and January, February and March (a period of drought), with a strong association between bacterial taxa and specific nutrients (dry matter, protein and structural carbohydrates) and climate variables (rainfall and temperature). Our study supports the hypothesis that diversity and community structure of the faecal microbiota of horses kept on pasture varies over a 12-month period, and this variation reflects changes in the nutrient composition of the pasture, which in turn is influenced by climatic conditions. The findings of this study may have implications for grazing management and the preparation of conserved forages for those horses susceptible to perturbations of the hindgut microbiota.

6.2 Introduction

Horses thrive on high-forage diets due to the microbial fermentation of fibre and other compounds in the hindgut (Janis, 1976). This digestive strategy requires a high food intake level (above 30 g/kg body weight$^{0.75}$ /day (Clauss *et al.*, 2014)) to maintain proper gastrointestinal function, with forage comprising the major component of the diet. Below this threshold level of forage intake, nutrient supply to the hindgut microbiota becomes the major constraint of digestive function and efficiency (Clauss *et al.*, 2014). Research has shown that the balance of microbial populations in the hindgut is important to maintain digestive health, immune function, and performance of the animal (Sadet-Bourgeteau and Julliand, 2010, Costa and Weese, 2012). Over the past decade, research has focused on understanding the structure and composition of these microbial communities in the equine hindgut (using caecal and faecal samples) (Daly *et al.*, 2001, Costa and Weese, 2012). Several studies have indicated that the structure of the equine hindgut microbiota is complex, and is comprised of a highly diverse community dominated by bacteria, amongst other microbial species such as archaea, protozoa and fungi (Daly *et al.*, 2001, Dougal *et al.*, 2013, O' Donnell *et al.*, 2013, Fernandes *et al.*, 2014).

Some studies have indicated that changes observed in the relative abundance of faecal bacteria may be associated with dietary modifications and gastrointestinal disturbances (Vörös, 2008, Costa *et al.*, 2012, Dougal *et al.*, 2014), which theoretically also occur prior to the onset of life-threatening conditions like colic and laminitis (Milinovich *et al.*, 2010, Moreau *et al.*, 2014, Weese *et al.*, 2015). Colic and laminitis are two of the most common problems that affect horses (Anonymous, 2000), so the potential to predict the onset of sub-clinical gastrointestinal disturbances by examining changes in the abundance of faecal bacteria has been suggested as a method which could perhaps prevent the sudden onset of clinical signs of disease (Weese *et al.*, 2015).

A previous study conducted by our group (Fernandes *et al.*, 2014) reported that the faecal bacterial community of forage-fed horses was highly diverse, and the profile was diet specific with significant differences observed in the relative abundance of certain bacterial genera (the most dominant one in both diets was an unclassified genus within the family Ruminococcaceae). Furthermore, when horses housed indoors and maintained on a commercial chopped ensiled forage were moved outside to graze on pasture, alterations in the

bacterial community profile were observed within four days. Although the gut microbiota of the horses appeared to adapt quickly to the new (pasture) diet, some fluctuation (both an increase and/or decrease) in the beta diversity of bacterial communities were observed over the 3-week study period, which was hypothesised to be due to changes in pasture composition (Fernandes *et al.*, 2014).

While many horses around the world are stabled and fed formulated compound diets, the major feed source for New Zealand horses is pasture, mostly consumed directly from the paddock, but also after conservation as ensiled forages and hay (Hoskin and Gee, 2004, Rogers *et al.*, 2007, Verhaar *et al.*, 2014). A temperate climate enables continuous growth of pasture in New Zealand, and many horses graze on pasture all year round (Fernandes *et al.*, Hoskin and Gee, 2004, Rogers *et al.*, 2007, Verhaar *et al.*, 2014). While there is vegetative grass-leaf throughout the year, the proportion of dead-leaf increases during late-summer (February) and autumn (March-April), and during plant stress due to drought or freezing temperatures (Litherland *et al.*, 2002, Longland and Byrd, 2006). Thus, there are seasonal changes in the dry matter (DM) and macronutrient composition of the pasture. While one study used conventional microscopic enumeration to compare the faecal microbiota of horses kept on summer versus winter pastures in Japan (Kobayashi *et al.*, 2006), there have been no studies that have used metagenomic techniques to examine the dynamics of bacterial communities in the faeces of horses maintained on pasture.

Given the expected seasonal fluctuations in pasture composition, we hypothesised that there is an effect of time (month or season) on the profile of the faecal bacterial community of pasture-fed horses. The aims of the current study were to investigate changes in the structure and composition of faecal bacterial communities over a 12-month period, by collecting cross-sectional snapshot data at monthly intervals, and to correlate the changes in microbiota populations with the macronutrient composition of pasture over the study period.

6.3 Materials and Methods

6.3.1 Ethics statement

The animals used in the study were part of Massey University's teaching herd. All faecal samples were collected during the routine per-rectal examinations organised to teach students enrolled in the undergraduate veterinary degree program. The use of animals,

including the welfare, husbandry and handling complied with the code of ethical conduct for the use of live animals for research, testing and teaching (Massey University Animal Ethics Committee, Palmerston North, New Zealand; Teaching Protocol number 11/100).

6.3.2 Experimental design

6.3.2.1 Animal details and management

Ten mares (eight Standardbred and two Thoroughbred, median age 15 years, interquartile range (IQR) 12–19 years), managed as a cohort at the Veterinary Large Animal Teaching Unit, Massey University, were enrolled in the study from April 2013 to March 2014. At the beginning of the trial, the mares had a mean body weight of 494 ± 44 kg, a median body condition score (BCS) of 5 (IQR 5-6) measured on a 9-point scale (Henneke *et al.*, 1983), and a median cresty neck score (CNS) of 2 (IQR 1-3) measured on a 6-point scale (Carter *et al.*, 2009). The mares were barren (non-pregnant) and were not "in work" during the study period. The horses were set stocked in a paddock that contained a standard New Zealand pasture mix of predominantly ryegrass and white clover species [~80-95% perennial ryegrass (*Lolium perenne*) and 5-20% white clover (*Trifolium repens*)] (Hoskin and Gee, 2004). The average sward height of the pasture was 4 ± 1 cm at the start of the study. The horses received unrestricted access to pasture (*ad libitum*) throughout the year, and were kept in three similar paddocks (~2.5 hectare) rotated every 5-6 months. Due to seasonal reduction in pasture growth from June to October, the diet was supplemented with hay (ryegrass-clover hay mix), which was offered to the horses in the paddock twice daily at the rate of 6-10 kg DM/horse/day. The hay was harvested from a single location (Manawatu, New Zealand) in January 2013, processed as one batch, and stored in a barn for use during the study. The horses had *ad libitum* access to water in self-filled troughs in the paddock. Faecal egg counts were performed on all horses every 4-6 weeks, followed by administration of individual dose-dependent anthelmintic treatments if necessary (Equitak Paste, Bayer Animal Health, New Zealand).

6.3.3 Sample collection

6.3.3.3 Faecal sample collection

A faecal sample was collected from each horse at monthly intervals between 1000 and 1200 hours on a single day. The faecal material were collected per-rectum, a sub-sample was transferred into a 2 ml polyethylene cryogenic vial (Ray Lab Ltd., Auckland, New Zealand),

and snap frozen in liquid nitrogen immediately. The samples were transferred to a -80°C freezer within two hours of collection and stored until laboratory analysis. A total of 120 faecal samples (10 horses × 12 months) were collected during the study period.

6.3.3.1 Feed sample collection

Representative pasture samples were collected from the paddock each month, on the same days as the faecal samples were collected. According to previously described pasture sampling techniques (Litherland *et al.*, 2002), the grazing behaviour of the horses was observed to identify sites used to estimate the grazing height of the pasture (height of stubble remaining after grazing). Within the paddock, fifty pasture-sampling sites were selected by walking in a zigzag manner through the paddock and selecting a site every 20 steps. At each site, approximately 20-50 g of pasture was cut (at the pre-estimated grazing height of ~1 cm above the ground) and collected in a polythene bag that was placed in an icebox. After collecting approximately 1 kg of pasture, the sample was mixed thoroughly and transferred to a zip-lock plastic bag. During the months from June-October, representative hay samples (500 g) were collected by taking multiple grab-samples from each hay bale (core and outer surface), which were also mixed thoroughly before transfer to zip-lock plastic bags. All samples were weighed (recorded as fresh weight), and stored at -20°C until further processing within 2-4 hours of collection. At six-monthly intervals, the samples were lyophilised (FD18, Cuddon Freeze Dry, Blenheim, New Zealand) and ground to pass through a 1 mm screen (Cyclotec 1093 Sample Mill, Foss, Hillerod, Denmark) (Pelletier *et al.*, 2010, Udén, 2010). The ground samples were stored at -20°C until laboratory analysis to evaluate the nutrient content of the feed samples, which was performed at the end of the study period.

6.3.4 Laboratory analysis

6.3.4.1 DNA extraction, amplicon library construction and sequencing

Nucleic acids were extracted from 100 mg of each faecal sample (n=120) using a combined bead-beating, phenol-chloroform and column purification protocol and QIAquick 96 PCR purification kit (Qiagen, Hilden, Germany) (Rius *et al.*, 2012, Kittelmann *et al.*, 2014), with some modifications (Appendix D-1, Text S1). The extracted and purified gDNA was eluted in 80 µl elution buffer (10 mM Tris; pH 8.5 with HCl). All gDNA samples were quantified using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality was assessed by visualisation of high

molecular weight bands on a 1% (wt/vol) agarose gel. A subset of samples (n=12) were quantified with Quant-iT dsDNA HS, RNA and Protein assay kits (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 Fluorometer (Invitrogen) to assess the quality of gDNA and to check for the presence of RNA and protein contamination.

The samples were normalised to 5 ng/µl gDNA per sample and bacterial 16S rRNA gene libraries were constructed using the Illumina two-step PCR library preparation method (Appendix D-1, Text S1; New Zealand Genomics Limited, Massey Genome Service, Palmerston North, New Zealand). Briefly, for each gDNA sample, the V3-V4 hypervariable region of the 16S rRNA gene was targeted using the universal primer pair S-D-Bact-0341-b-S-17; S-D-Bact-0785-a-A-21 (Klindworth *et al.*, 2012), because these sub-regions are the most reliable regions for representing the full-length 16S rRNA sequences, with superior phylogenetic resolution of most bacterial phyla (Yu and Morrison, 2004, Yang *et al.*, 2016). The amplicons were attached to the Illumina adapter overhang nucleotide sequences (forward and reverse) in the amplicon-PCR step. This was followed by ligation with a unique 8 bp dual-index barcode sequence (Nextera XT DNA library preparation kit, Illumina, San Diago, CA, USA) in the index-PCR step, for individual sample identification (Appendix D-2, Table S1). Amplification was performed on a Thermocycler ProS (Eppendorf, Hamburg, Germany), with an initial denaturation at 95°C for 3 min, 25 cycles of denaturing (95°C for 30 s), annealing (55°C for 30 s) and elongation (72°C for 30 s), and a final 5-min extension at 72°C. At each PCR step amplicons were generated using a KAPA HiFi PCR kit (KapaBiosystems, Wilmington, MA, USA), purified using a magnetic bead capture kit (AMPure, Agencourt, Beckman Coulter, Beverly, MA, USA), and quantified on a Qubit 2.0 Fluorometer (Invitrogen), to check the quality of gDNA (Quant-iT dsDNA HS assay kit, Invitrogen) and the presence of contamination (Quant-iT RNA and Protein assay kits, Invitrogen). Following validation of the purified sequence libraries using a DNA 1000 labchip on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), the 16S metagenomic sequence libraries (n=120) were pooled in equimolar concentrations into two pools with 60 libraries each. The pooled libraries were denatured using fresh NaOH, and spiked with 10% volume of a PhiX control library (PhiX control kit v3, Illumina), before loading onto 2 × 250 base paired-end sequencing runs (60 libraries per run, samples from January-June on run 1 and July to December on run 2) using the Illumina MiSeq platform (MiSeq 500 cycle kit, v2 chemistry, Illumina) (Bartram *et al.*, 2011, Kozich *et al.*, 2013, Fadrosh *et al.*, 2014).

6.3.3.2 Analysis of nutrient composition

The pasture (n=12) and hay (n=5) samples were processed using analytical chemistry methodologies (Nutrition Laboratory, Massey University, New Zealand) for the quantitative determination of Dry Matter (DM) (AOAC 930.15; convection oven 105°C, Contherm 2000, Contherm Scientific Ltd., Lower Hutt, New Zealand), and Ash (AOAC 942.05; Furnace 550°C, Elecfurn:Muf 25/20/40, The Electric Furnace Co Ltd., Auckland, New Zealand). Total Nitrogen (N) was determined by the Dumas method (Dumas, 1831) and was converted to Crude Protein (CP) by multiplying by 6.25 (AOAC 968.06; CNS 2000, LECO Corporation, St. Joseph, MI, USA). Fat was determined by the Soxhlet extraction method (AOAC 991.36; Tecator Soctec System HT, 1043, FOSS, Hillerod, Denmark). Fibre analysis including Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Lignin was determined using the ANKOM TDF Fiber Analyser (ANKOM Technology, Macedon, NY, USA) and Hot Water Soluble Carbohydrate (HWSC) content was determined by the Nelson-Somogyi assay (Nelson, 1944, Somogyi, 1952). The Gross Energy (GE) content was determined through heat of combustion (Gallenkamp Adiabatic Bomb Calorimeter, Loughborough, Leicestershire, United Kingdom). Other feed values were calculated as follows: Total carbohydrates (CHO) = 100 - (CP + fat + ash); non-structural carbohydrates (NSC) = 100 - (CP + fat + ash + NDF); and Digestible energy (DE MJ/kg DM) = 2118 + 12.18 (CP) – 9.37 (ADF) -3.83 (NDF-ADF) + 47.18 (fat) + 20.35 (NSC) – 26.3 (Ash) × 0.00418 (Pagan, 1998, Hall, 2003).

6.3.5 Data recording and analysis

6.3.5.1 Data recording

The general health, feeding and management of the horses was monitored daily by the farm manager. During the monthly collection of faecal samples for microbiota analysis, a subjective assessment of body condition and cresty neck scores was made by one of the authors (KAF). The pasture cover was also monitored on a monthly basis by measuring the average sward height (cm ± SD) of the pasture by using a standard metric ruler in the areas being grazed by the horses at the time of faecal and feed sample collection, The recorded sward height was compared with a custom scale (Farmax sward stick, Hamilton, New Zealand) to estimate the pasture herbage mass (kg DM/ha) available to the horses. Daily recordings of temperature and rainfall were retrieved from the National Climate Database (National Institute of Water and Atmospheric Research-NIWA, New Zealand), which contained data from a

weather station situated ~2 km from the paddock location (Palmerston North EWS, coordinates 40.38195 S 175.60915 E, AgResearch Grasslands Ltd.). The data for temperature and rainfall were averaged to present monthly recordings.

6.3.5.2 Bioinformatics analysis

Quality control analysis was performed on the original sequences using three processes: SolexaQA++ (*http://solexaqa.sourceforge.net/*), FastQC (*http://www.bioinformatics.babraham.ac.uk/projects/fastqc/*) and FastQscreen (*http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/*).

The raw sequence reads obtained from the Illumina MiSeq runs were aligned against the PhiX genome using BWA (*http://bio-bwa.sourceforge.net/*), and the PhiX sequences detected were removed, leaving the unaligned sequences that were included in further downstream analysis. The SAM (Sequence Alignment/Map) files generated from BWA and the fastq files were reconstructed using SAM Tools (available at *http://broadinstitute.github.io/picard/*). Illumina adaptors and PCR primers were removed using the FASTQ processing utilities "fastq-mcf" program (available in the ea-utils suite of tools version 1.1.2-621, *https://code.google.com/p/ea-utils/*). The sequence reads were assigned to corresponding samples by examining the 8 bp barcode sequence (Kozich *et al.*, 2013), and the read pairs were extracted and concatenated according to the barcodes for each paired read from each sample, by joining together the overlapping reads using the FLASh software (version 1.2.11, *http://ccb.jhu.edu/software/FLASH/*), which generated the best aligned contigs (Magoč and Salzberg, 2011). The processed sequences were trimmed to their longest contiguous segment for which error probabilities were greater than a threshold of 0.003 (equivalent to quality of ~25 Phred score) using the DynamicTrim application from the SolexaQA++ software (version 3.1.2, *http://solexaqa.sourceforge.net/)* and short reads (<250 bp) were removed from the bacterial sequence library using the LengthShort application (Cox *et al.*, 2010). All sequences that did not meet the above quality filtering criteria were excluded from further downstream analysis. The project is registered with NCBI PRJNA286058, and the sequence data generated in this study are available via the Sequence Read Archive under the accession number SRA272143.

Ecological analysis on the retained sequence data were performed using the QIIME package (Quantitative Insights Into Microbial Ecology, v1.8) (Caporaso *et al.*, 2010b). Clustering of operational taxonomic units (OTUs) was performed using the uclust method at

a 97% similarity threshold and potential chimeras were removed using the usearch61 option in the QIIME scripts (*pick_otus.py* –m usearch61) (Edgar, 2010). Representative OTU sequences were aligned using PyNAST (Caporaso *et al.*, 2010a) and assigned to taxonomic ranks (McDonald *et al.*, 2011) by a BLAST-search against the Greengenes core reference alignment database for bacterial 16S rRNA genes (DeSantis *et al.*, 2006).

To access the richness of bacterial taxa captured within the samples, Collector's curves were constructed from the original OTU tables generated in QIIME, using the *alpha_diversity.py* script and the "observed species" metric. The alpha diversity rarefaction analysis was computed for a maximum of 19,250 sequences per sample, and was visualised for each parameter included in the metadata (Appendix D-2, Table S1). Subsequently, the original OTU table was rarefied to a subsample of 19,250 sequences per sample, in an attempt to decrease bias caused by non-uniform sequencing depth (Gihring *et al.*, 2012). Good's coverage (mean percentage ± SD) was estimated to ensure representative subsampling (Good, 1953), which was calculated in QIIME using the *alpha_diversity.py* script with the "goods coverage" metric and summarised using MS Excel (version 2010, Microsoft Corp., Redmond, WA, USA).

Alpha diversity was evaluated at the genus level using the PAST software (version 3.08, *http://folk.uio.no/ohammer/past/*) (Hammer *et al.*, 2001), and included the Simpson's index of diversity (Simpson, 1949), Shannon-Wiener index of entropy (Spellerberg and Fedor, 2003) and the Chao1 index for richness of bacterial genera (Gotelli and Colwell, 2011). Relative abundance of bacterial taxa were summarised at phylum and genus levels. Bacterial phyla and genera with relative abundances <1% in all samples were grouped as "other phyla" and "other genera", respectively. The taxonomic profiles for the bacterial phyla and genera were visualised on heatmaps using MetaPhlAn (version 1.7.8, *http://huttenhower.sph.harvard.edu/metaphlan*) (Segata *et al.*, 2012). Beta diversity was evaluated on a genus level using the QIIME pipeline (Caporaso *et al.*, 2010b), and included only those bacterial taxa that represented ≥1% of the total community, in at least one sample.

Differences between bacterial communities were determined using Bray-Curtis dissimilarity, which takes into account the presence or absence of a species and the relative abundance. Principal coordinate analysis (PCoA) was performed in QIIME and the clustering of samples based on the first three principal coordinates was visualised using EMPeror (Vazquez-Baeza *et al.*, 2013), SigmaPlot (version 13, Systat Software, Inc., San

Jose, CA, USA) and MEGAN5 MEtaGenome ANalyzer (version 5, *http://ab.inf.uni-tuebingen.de/software/megan5/*), as required. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was performed in QIIME, based on the Bray-Curtis dissimilarity index, to visualise the hierarchical clustering of samples by season and month. The dendrograms were visualised using MEGA6 (version 6.0) (Tamura *et al.,* 2013) and MEGAN5 software tools.

6.3.5.3 Statistical analyses

The data generated in QIIME and PAST were imported into MS Excel, and re-formatted where necessary, before tests for statistical significance were conducted. Statistical analysis was performed using the SAS v9.4 (SAS Institute Inc., Cary, NC, USA), STATA v12.1 (Stata Corp., College Station, TX, USA), and R (www.r-project.org) software packages. All variables were checked for normal distribution using the Shapiro-Wilk test (significance value of $P<0.05$). All data are presented as mean ± SD for parametric data or median ± IQR for non-parametric data. Seasons were classified as follows: Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November; and Summer – December, January, February.

Differences for the alpha diversity indices between groups (diet, horse, month, and season), and for the median Bray-Curtis dissimilarity indices between the within- and between-horse variation in each diet group and between-horse variation within each diet, were determined using the Kruskal-Wallis test with a Steel-Dwass correction for multiple sample comparisons (as required) at a significance value set at $P<0.05$ (proc npar1way Wilcoxon dscf in SAS). The effect of diet, horse, season, and month on the beta diversity of bacterial taxa were tested using the Analysis of Similarity (ANOSIM) option with the QIIME script *compare_categories.py*. Significant differences between the relative abundance of bacterial taxa were determined by the *group_significance.py* script in QIIME using the Kruskal-Wallis test with Bonferroni adjustment for multiple comparisons (adjusted P values were $P<0.003$ for phylum level and $P<0.001$ at genus level comparisons).

Correlations between the four most dominant bacterial taxa with nutrient and climate variables were determined by Pearson's Correlation with P values that were calculated and plotted in R using the packages hmisc (*http://cran.r-project.org/web/packages/Hmisc*) and corrplot (*http://cran.r-project.org/web/packages/corrplot*). For the correlation analysis of nutrient variables versus

microbiota abundance, data on the nutrient composition of pasture from only those months when pasture was the sole component of the diet were included. The four dominant bacterial genera selected were renamed as follows: Genus1 (unclassified genus within family Ruminococcaceae), Genus2 (unclassified genus within order Bacteroidales), Genus3 (unclassified genus within order Clostridiales) and Genus4 (unclassified genus within family Lachnospiraceae).

6.4 Results

6.4.1 Health and condition of the horses

The median BCS of the horses during the study was 5 (IQR 4-6) (P=0.96) and the median CNS was 2 (IQR 1-3) (P=0.99), with no significant differences observed during the 12-month study period. The horses remained clinically normal throughout the study period. There were no reports of gastrointestinal or musculoskeletal issues during the experimental timeframe and none of the horses received antibiotic treatment during the study period.

6.4.2 Forage and climate monitoring

The macronutrient composition of the pasture and hay samples recorded at monthly intervals during the 12-month study period is shown in Table 6.1. The nutrient composition of pasture varied during the study period. With rising temperatures and rainfall in spring (September- November), the sward height and pasture cover increased, the %HWSC increased to the highest values in October-November, and %NSC values peaked in late-spring (October-November) and early-summer (December). An increase in NSC was also observed in early-autumn (March) following the drought period in summer (January-February). The %DM in pasture was the highest in February (late-summer) and March (early-autumn), which coincided with the lowest rainfall and highest temperatures recorded during the drought period. Correspondingly high values for %CHO, %NDF and %ADF content and low values for %CP and %HWSC content were also observed in pasture during this drought period (January-March) (Table 6.1).

The %DM in hay was relatively consistent (ranging from ~95 to 96% DM), but there was some variation in the %CP (ranging from ~9-15%) observed across the five sampling time-points in the same batch of hay (Table 6.1). There was also variation in the %HWSC (~6-

9%), %NDF (~43-63%), %ADF (~30-37%) and %NSC (~16-30%). However, the variation observed in the hay samples was lower than the variation observed in the pasture samples (Table 6.1).

Table 6.1. Nutrient composition of pasture and hay and climate variables measured over a period of 12 months.

Variables	Diet	Apr 2013	May 2013	Jun 2013	Jul 2013	Aug 2013	Sep 2013	Oct 2013	Nov 2013	Dec 2013	Jan 2014	Feb 2014	Mar 2014
Nutrient composition													
DM (%)	P	21.7	17.6	17.4	17.0	18.5	19.1	23.5	20.2	20.9	26.6	44.6	52.0
	H			94.4	95.7	96.3	96.0	96.3					
CP(%)	P	21.0	24.2	26.9	27.5	24.7	21.9	25.5	21.1	28.2	19.9	15.1	13.2
	H			15.0	9.3	11.1	8.8	9.8					
Fat (%)	P	3.2	3.6	3.8	3.4	3.3	3.6	3.9	3.7	4.4	3.3	2.3	2.1
	H			1.4	1.4	1.4	1.5	1.5					
CHO‡ (%)	P	67.0	58.5	58.1	55.0	54.7	61.1	65.7	70.5	61.3	72.4	77.6	79.7
	H			76.2	82.4	79.2	81.9	80.4					
Ash (%)	P	13.0	17.8	15.3	17.8	20.5	18.0	10.1	9.7	11.2	10.0	10.2	9.2
	H			11.0	8.6	9.1	8.9	9.2					
HWSC (%)	P	8.9	10.6	11.4	10.1	10.1	11.6	13.4	14.7	9.1	8.4	7.5	8.2
	H			9.7	8.8	6.6	7.4	6.4					
NDF (%)	P	52.6	46.2	46.2	46.1	43.7	45.6	43.5	38.7	39.1	52.4	58.0	58.7
	H			43.1	58.8	58.9	62.4	63.4					
ADF (%)	P	28.6	27.6	25.8	26.8	28.7	27.3	22.3	20.6	22.6	27.5	31.3	31.8
	H			30.2	34.6	36.2	36.5	36.8					
ADL (%)	P	2.9	6.4	3.9	6.6	9.5	5.9	0.9	1.0	0.8	0.9	1.1	0.7
	H			0.3	0.5	0.5	0.4	0.5					
NSC§ (%)	P	10.1	8.2	7.8	5.2	7.8	10.8	17.0	26.8	17.1	14.5	14.4	16.8
	H			29.5	21.9	19.5	18.4	16.3					
GE (KJ/g)	P	18.5	17.7	18.3	17.7	16.8	17.3	19.1	19.0	19.3	18.7	18.2	18.3
	H			17.8	17.8	18.1	18.0	17.9					
DE† (MJ/kg DM)	P	8.1	7.8	8.3	10.0	10.8	10.2	9.2	8.5	8.7	8.5	8.2	8.6
	H			9.8	8.8	8.6	8.3	8.1					

Variables	Diet	Apr 2013	May 2013	Jun 2013	Jul 2013	Aug 2013	Sep 2013	Oct 2013	Nov 2013	Dec 2013	Jan 2014	Feb 2014	Mar 2014
Pasture variables													
Sward height (cm)		3	4	4	4	4	4	5	7	7	6	4	4
Pasture cover (kgDM/ha)		1290	1540	1300	1300	1300	1580	1780	2330	2500	2240	1710	1540
Climate variables													
Mean daily Rainfall (mm)		3.7	1.1	2.8	1.8	1.9	4.6	3.9	2.1	2.2	1.9	0.8	0.4
Mean Temperature (max °C)		20	16	14	14	15	16	18	21	22	22	24	22

Legend: P- pasture, H-hay; DM - dry matter; CP – crude protein; CHO – total carbohydrates; HWSC - hot water soluble carbohydrates;NDF - neutral detergent fibre;ADF - acid detergent fibre; ADL - acid detergent lignin;NSC - non-structural carbohydrates;GE - gross energy;DE - digestible energy.

[‡]Total carbohydrates (CHO) = 100 - (CP + fat + ash)

[§] Non-structural carbohydrates (NSC) = 100 - (CP + fat + ash + NDF)

[†]Digestible energy (DE) = 2118 + 12.18 (CP) – 9.37 (ADF) -3.83 (NDF-ADF) + 47.18 (fat) + 20.35 (NSC) – 26.3 (Ash)

6.4.3 Comparison of the faecal microbiota (bacterial communities)

6.4.3.1 Metrics of sequencing data and rarefaction analysis

The two runs on the Illunima MiSeq platform generated ~16 million sequences. On preliminary examination of the data, one sample (collected from Horse 6 during the month of August) had a significantly lower number of sequences (n=2,808 reads) than other samples (n≥19,250 reads). On preliminary phylogenetic analysis, another sample (collected from Horse 9 during the month of May) had a significantly higher proportion (96.9%) of proteobacteria when compared to the other samples (0.1-1.6%). These two samples were outliers, and hence, were removed from downstream phylogenetic analysis (dataset of n=118 faecal samples). The mean number of sequences per sample and the metrics of data for sequences that passed quality filtering are shown in Table 6.2. After normalisation at 19,250 sequences per sample, the total number of OTUs detected at 97% similarity across all samples was 85,725. The mean number of OTUs per sample was 4,819 (range 3,521 – 6,499). A total of 2,271,500 bacterial sequences obtained from the samples (n=118) were included in the downstream analysis, wherein at least 33 bacterial phyla were detected, which encompassed at least 90 different taxonomic classes, 170 orders, 323 families and 646 genera.

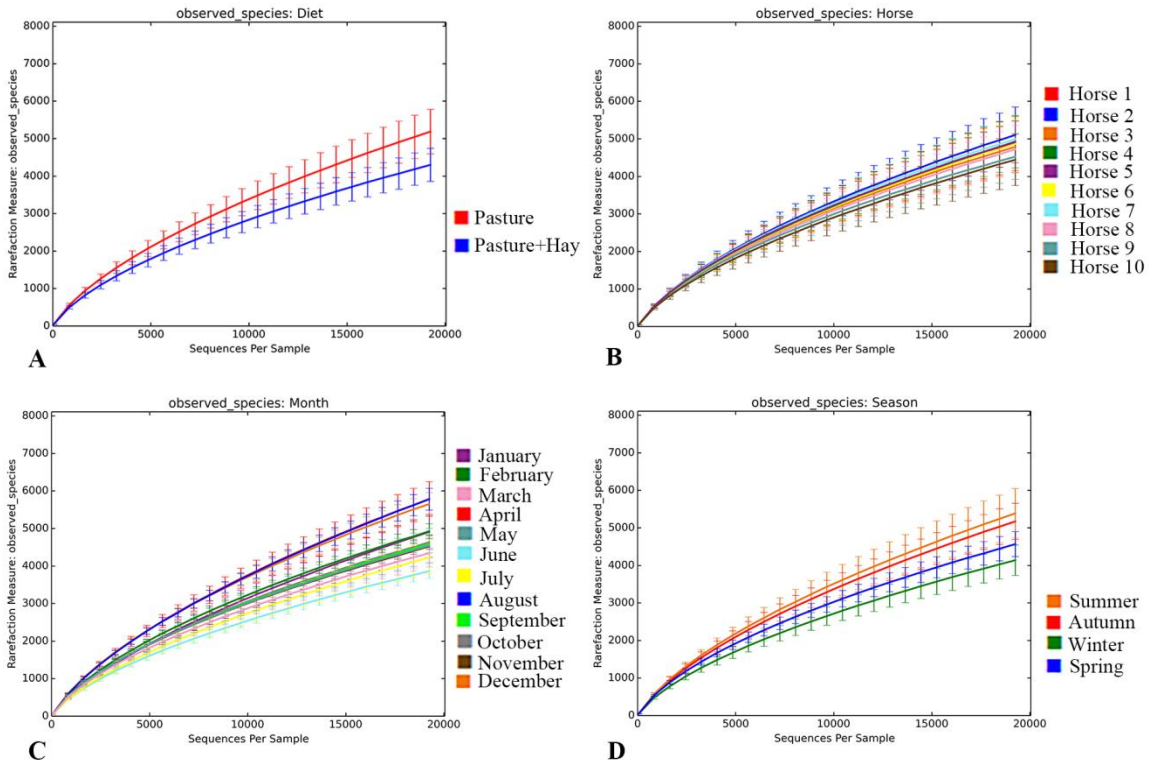
Table 6.2. Metrics of sequencing and quality screening.

Details	Bacterial sequences
Initial QC processed reads[§]	
Total number of reads	16,075,202
Mean number of reads per sample	117,448
(range)	(47,154 – 189,708)
High quality reads used in downstream analysis[†]	
Total number of reads	5,257,753
Mean number of reads per sample	44,557
(range)	(19,250 – 73,405)
Mean length of reads (bp)	356
(range)	(250 – 486)
Total OTUs detected at 97% similarity	123,645

[§]After using SolexaQA++, fastaQC, fastQscreen, BWA PhiX, fastq-mcf, flash
[†]After using DynamicTrim, LengthShort, Chimera check

The rarefaction curves generated using the observed species metric for alpha diversity of the bacterial sequences are displayed in Figure 6.1. New OTUs were identified at the end of sub-sampling at 19,250 sequences per sample, which may indicate a lack of complete sampling effort. However, the rate of new OTU discovery was relatively limited around that sub-sampling threshold. Good's coverage estimates on the normalised OTU table indicated that the sampling depth had adequately captured a large part of the OTU diversity in all samples, with the mean coverage being 84 ± 3%. Figure 6.1 also indicated that there were some differences in the diversity within the faecal bacterial community for diet, season, and month.

Figure 6.1. Alpha rarefaction curves illustrated by diet, horse, month and season.



Legend: Each rarefaction curve was generated using the observed species metric for up to 19,250 sequences per sample. The panels represent the rarefaction curves for diet (A), horse (B), month (C) and season (D). The diet periods were categorised as "Pasture" when the horses were grazed exclusively on pasture, and "Pasture+Hay" when the horses were grazed on pasture and supplemented with hay in the paddock. Seasons were categorised as follows: Summer – December, January, February; Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November.

6.4.3.2 Diet-specific differences in diversity of the faecal bacterial community

The Pasture and Pasture+Hay diets showed high diversity of bacterial genera detected in the faeces. The Simpson's and Shannon-Wiener diversity indices were significantly higher (P<0.001) during the months when horses were kept exclusively on pasture versus pasture supplemented with hay. This was reflected in the trend for differences in the richness of bacterial genera (Chao1) between the two time-periods (Table 6.3).

Table 6.3. Comparison of alpha diversity between diets.

Diversity indices	Pasture		Pasture + Hay		P value
	Median	IQR	Median	IQR	
Simpson's (diversity)	0.90	(0.89 - 0.91)	0.89	(0.88 - 0.90)	<0.001*
Shannon-Weiner (entropy)	3.02	(2.93 - 3.12)	2.91	(2.84 - 2.96)	<0.001*
Chao1 (richness)	167	(157 - 184)	161	(145 - 179)	0.075

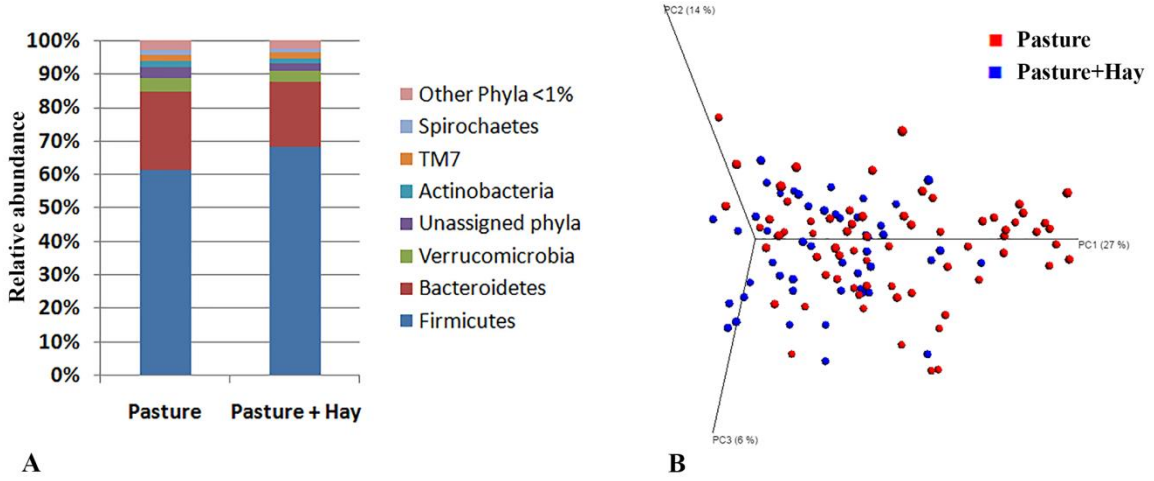
*Level of significance was P≤0.05, IQR – interquartile range

The bacterial community of both diets comprised 33 phyla, 14 of which were present at ≥1% abundance and the remaining 19 phyla (other phyla) had abundances <1% (overall 15 taxa for comparisons at the phylum level). The bacterial community was dominated by two phyla, the Firmicutes (Pasture 62% and Pasture+Hay 68%) and the Bacteroidetes (Pasture 23% and Pasture+Hay 19%), which together accounted for >80% of the overall abundance of bacterial phyla (Figure 6.2, panel A). A total of 646 bacterial genera were identified, 51 of which had abundances ≥1% and the remaining 595 genera (other genera) had abundances <1% (overall 52 taxa for comparisons at genus level). The most abundant bacterial genera in both of the diet periods were an unclassified genus within the family Ruminococcaceae (20-23%), an unclassified genus within the family Lachnospiraceae (13-14%), an unclassified genus within the order Clostridiales (12-14%) and an unclassified genus within the order Bacteroidales (9-11%), which accounted for ~54-62% of the overall abundance of bacterial genera.

There were significant effects of diet periods on the beta diversity of bacterial genera (ANOSIM, P=0.002, R^2=0.122), which appeared to cluster by diet period on the principal coordinate analysis, with 47% of the variation explained on three principal coordinates (27%, 14% and 6% on PCs 1, 2 and 3, respectively) (Figure 6.2, panel B). This clustering by diet

period was further described by significant differences observed in the relative abundances of seven bacterial phyla (Appendix D-3, Table S2) and 12 bacterial genera (Appendix D-4,Table S3).

Figure 6.2. Comparison of the faecal bacterial community across diet periods.



Legend: (A) Mean relative abundance of bacterial phyla identified in the faecal samples of horses fed Pasture versus Pasture+Hay. (B) 3-D graph of principal coordinate analysis (PCoA) showing beta diversity of the bacterial community in the faeces of horses fed Pasture and Pasture+Hay. The first three PCs explained 47% of the variation (27% by PC1, 14% by PC2 and 6% by PC3).

6.4.3.3 Inter- and intra-horse variation

There was a significant effect of horse on the beta diversity of bacterial genera (ANOSIM, P=0.002, R^2=0.067). The median Bray-Curtis dissimilarity index for the period when pasture was fed (0.21 [IQR 0.17-0.26]) was greater than the Pasture+Hay diet period (0.20 [IQR 0.17-0.24]) (P=0.001). This pattern was seen for between-horse (P=0.006) and within-horse (P=0.024), comparisons. Differences in the beta diversity observed between horses were reflected in the significant differences in the relative abundances of several bacterial taxa. At the phylum level, the relative abundances of only three less abundant phyla were significantly different between the horses (Appendix D-5, Table S4). The relative abundances of the most dominant phyla (Firmicutes and Bacteroidetes) did not differ. At the genus level, the relative abundances of two genera differed between the horses, and these did not include the most dominant genera (Appendix D-6, Table S5).

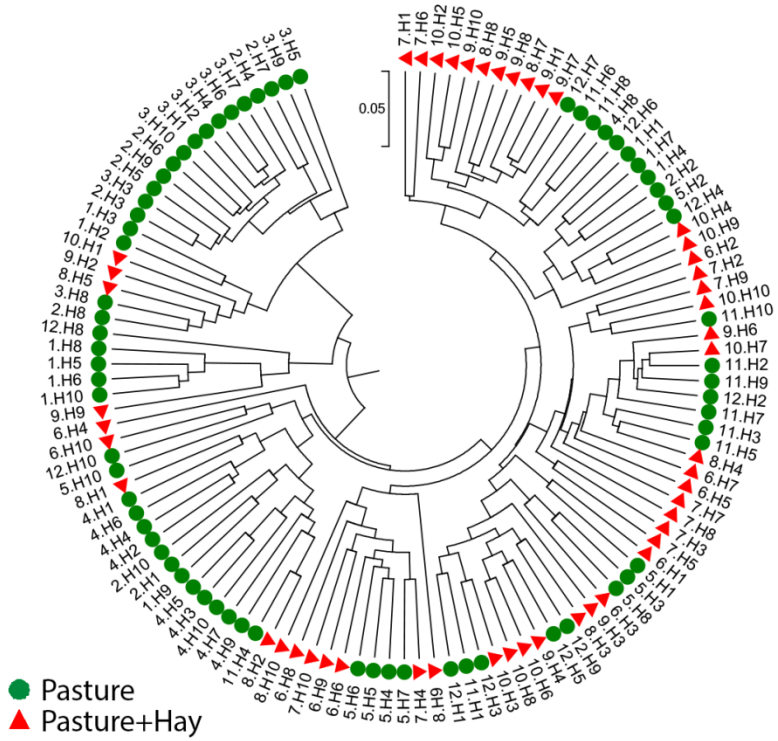
Within each diet period, there was a significant difference in the median Bray-Curtis dissimilarity index between individual horses (Pasture P<0.007 and Pasture+Hay P<0.001), indicating the presence of some variation in the bacterial communities between individual horses (Table 6.4). However, the median between-horse dissimilarity was similar to the median within-horse dissimilarity (Pasture P=0.758 and Pasture+Hay P=0.115), indicating that the significant effect of horse on the beta diversity of bacterial genera may be due to temporal factors. The UPGMA cladogram constructed using all samples included in the study shows some clustering of horses by diet periods, and indicates the possibility of effects due to temporal factors (Figure 6.3).

Table 6.4. Comparison of Bray-Curtis dissimilarity indices between the horses within each diet period.

	Pasture		Pasture+Hay	
Horse	Median	IQR	Median	IQR
1	0.21	(0.17-0.25)	0.20	(0.17-0.24)
2	0.18	(0.14-0.20)	0.18	(0.15-0.21)
3	0.19	(0.16-0.24)	0.23	(0.19-0.26)
4	0.21	(0.16-0.25)	0.23	(0.18-0.29)
5	0.21	(0.16-0.28)	0.18	(0.16-0.24)
6	0.23	(0.19-0.28)	0.20	(0.18-0.24)
7	0.19	(0.15-0.22)	0.17	(0.14-0.21)
8	0.22	(0.20-0.24)	0.21	(0.16-0.23)
9	0.21	(0.18-0.27)	0.22	(0.21-0.26)
10	0.22	(0.18-0.28)	0.24	(0.19-0.27)
P value	<0.007*		<0.001*	

*Level of significance was P≤0.05 with Steel-Dwass test for multiple comparisons, IQR – interquartile range

Figure 6.3. The bacterial community structure of faecal samples (n=118) included in the study.



Legend: The circular cladogram showing beta diversity of the faecal sampleswas constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The sample labels are coloured by diet periods and consist of a month number (1-12 representing Jan-Dec) and horse number (H1-H10).

6.4.3.4 Temporal effects on the diversity of the faecal bacterial community

All seasons and months showed high values for the alpha diversity of bacterial genera. There was a significant difference in the Simpson's diversity index between seasons, with significantly higher median diversities observed in autumn compared to summer, winter, and spring (Table 6.5). The median Shannon-Wiener indices were significantly higher in summer and autumn than in winter and spring, whereas the richness of bacterial genera (Chao1 index) was similar in summer, autumn and winter, but significantly lower in spring. Significant differences in alpha diversity indices were also observed between the 12 months of the study period (Table 6.6).

Table 6.5. Comparison of the median alpha diversity indices between seasons.

Diversity indices	Autumn	Winter	Spring	Summer	P value
Simpson's (diversity)	0.91[b]	0.89[a]	0.89[a]	0.89[a]	0.0001
Shannon-Wiener (entropy)	3.08[a]	2.93[bc]	2.91[c]	3.00[a]	0.0001
Chao1 (richness)	173[a]	169[a]	154[b]	173[a]	0.0002

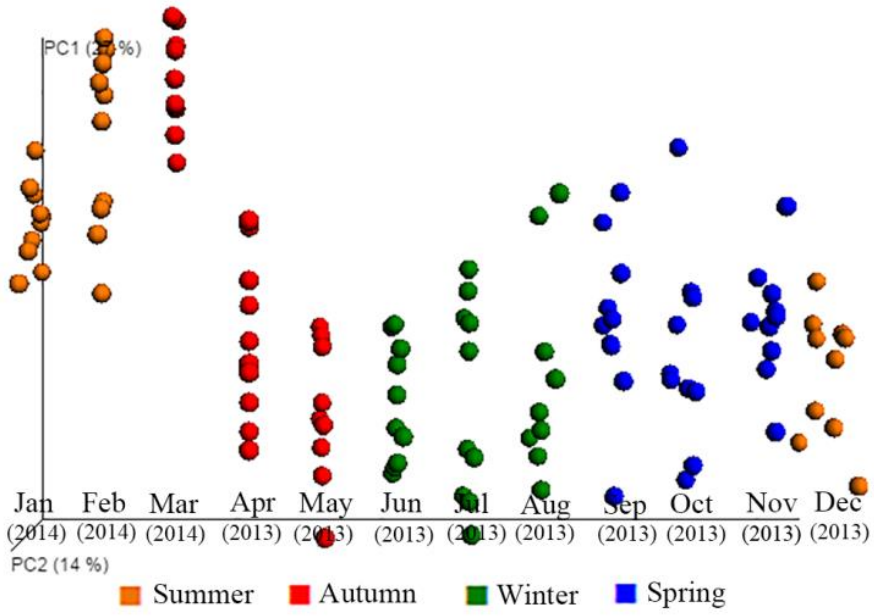
Legend: Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November; Summer – December, January, February. Different superscripts within a row represent significant differences (P<0.005).

Table 6.6. Comparison of the median alpha diversity indices between months.

Diversity indices	Apr 2013	May 2013	Jun 2013	Jul 2013	Aug 2013	Sep 2013	Oct 2013	Nov 2013	Dec 2013	Jan 2014	Feb 2014	Mar 2014	P value
Simpson's (diversity)	0.90	0.90	0.90	0.89	0.88	0.89	0.89	0.90	0.89	0.89	0.92	0.92	0.0001
Shannon-Wiener (entropy)	3.03	2.99	2.95	2.92	2.90	2.89	2.90	2.94	2.93	2.97	3.19	3.14	0.0001
Chao1 (richness)	162	169	171	187	142	148	165	144	162	162	192	181	0.0001

There were significant effects of season (ANOSIM, P=0.001, R^2=0.190) and month (ANOSIM, P=0.001, R^2=0.479) on the beta diversity of bacterial genera in the faeces. Although there appears some overlap on the PCoA graphs, clustering by season (Appendix D-7, Figure S1, panel A) and month (Appendix D-8, Figure S2, panel B) was observed, where most (~47%) of the variation was explained on three principal coordinate axes. Figure 6.4 illustrates the beta diversity of the bacterial genera detected in the faeces of the 10 horses, grouped by season. The dispersion of the individual samples on PC1 indicates that there was some variation in the beta diversity among the 10 horses. However, shifts in the beta diversity were evident across the seasons, with one cluster observed in the last three months of the study during the drought period (January, February, March), a second cluster during the late-autumn and winter months (May, June, July) and a third cluster comprising the remaining months of the year (Figure 6.4). The hierarchical clustering of the bacterial communities observed on PCoA was confirmed by the separation of clades observed for the three clusters on the UPGMA cladogram constructed for the samples grouped by month (Figure 6.5). The bacterial community structure in autumn and summer (dryer pasture) originated from a different clade to that of spring and winter (greener pasture) (Appendix D-8, Figure S2 and Appendix D-9).

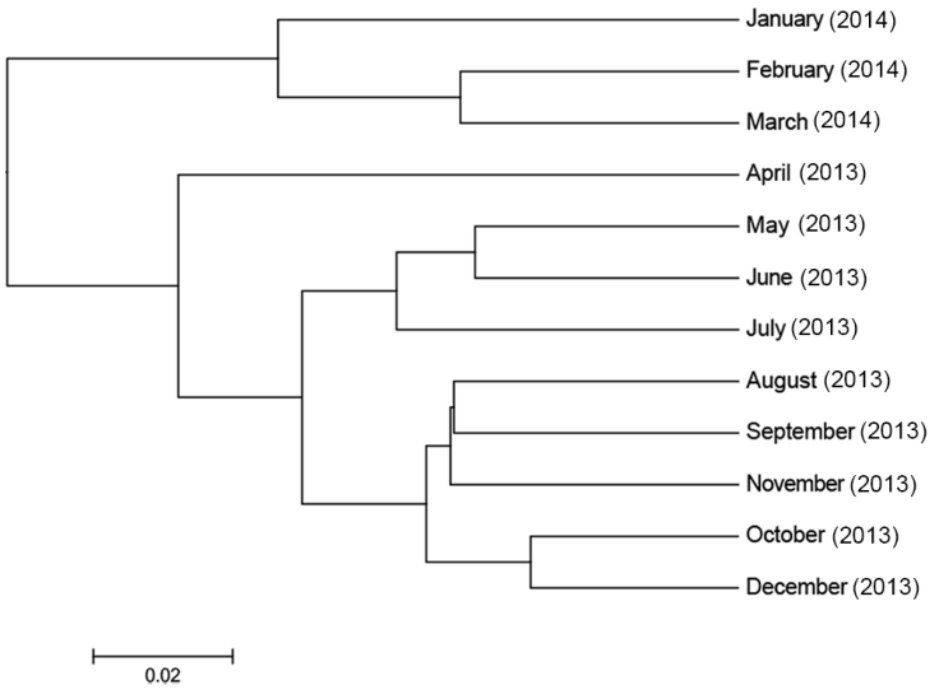
Figure 6.4. PCoA of the beta diversity of faecal bacteria in horses illustrated by season over a 12-month period.



Legend: The graph shows the principal coordinate analysis (PCoA) of the beta diversity in the bacterial community; with each faecal sample represented as a dot (i.e. each dot represents a

horse within a month). The principal coordinates explain 27% (PC1) and 14% (PC2) of the variation. The months of the year are shown on the horizontal axis and seasons are colour coded and categorised as follows: Summer – December, January, February; Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November.

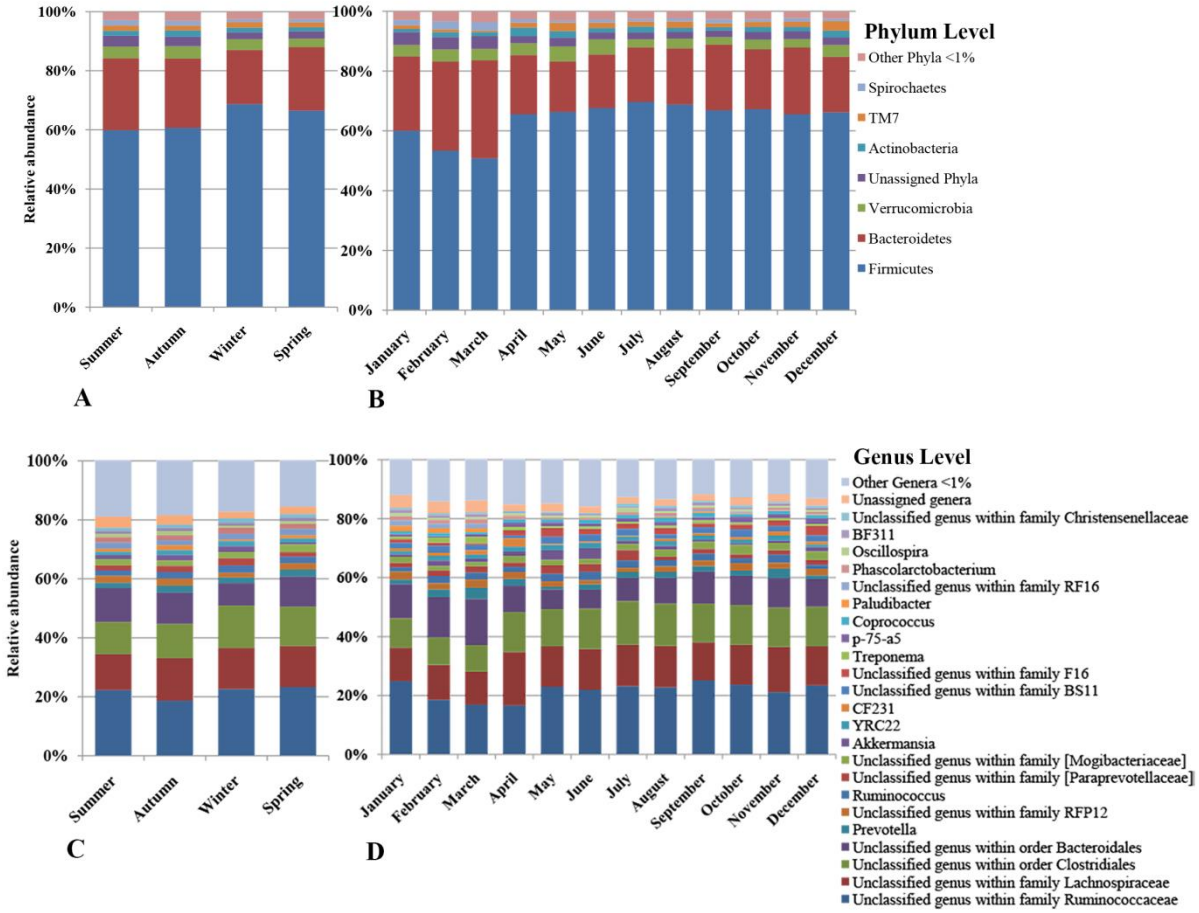
Figure 6.5. Month-wise hierarchial clustering of the faecal bacterial community of horses included in the study.



Legend: The cladogram shows the beta diversity of the bacterial community in the faecal samples and was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

The temporal effects and hierarchical clustering (by season and month) observed in the bacterial community structure were also described by differences in the relative abundances of several bacterial taxa at the phylum and genus levels (Figure 6.6). Across seasons, five bacterial phyla and 21 genera were significantly different (Appendic D-10, Table S6 and Appendix D-11, Table S7, respectively), while across months, 11 bacterial phyla and 39 genera were significantly different (Appendix D-12, Table S8 and Appendix D-13, Table S9, respectively). In both the season and month comparisons, significant differences were observed between most of the dominant bacterial phyla and genera.

Figure 6.6. Season- and month-wise comparison of relative abundances of faecal bacteria obtained from horses included in the study.



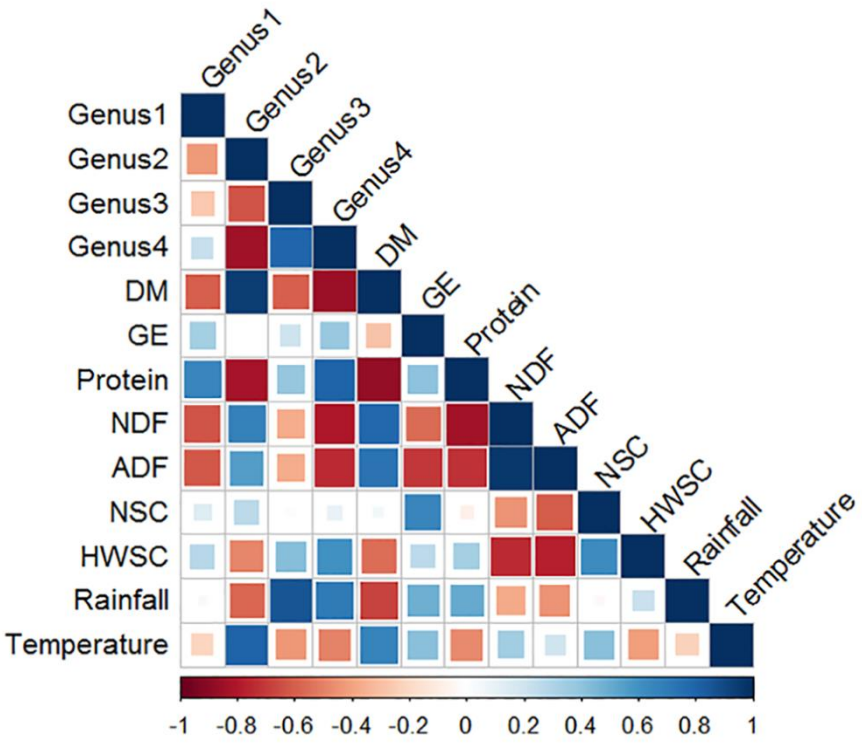
Legend: The relative abundances of the bacterial community are presented at phylum and genus levels. The stacked bar charts in panels (A) and (C) show the relative abundances by seasons, and panels (B) and (D) show the relative abundances by month. Phyla and genera with mean relative abundances of <1% were categorised as other phyla <1% or other genera <1%.

6.4.3.5 Correlation between nutrient composition of pasture, climate variables and relative abundance of faecal bacterial genera

Genus 2 (a genus within the phylum Bacteroidetes) was negatively correlated with Genera 1, 3 and 4 (genera within the phylum Firmicutes) (Figure 6.7). Genus 2 was positively correlated with temperature and pasture DM content, and negatively correlated with pasture protein and HWSC content, and rainfall. In contrast, Genera 1, 3, and 4 were negatively

correlated with pasture DM, NDF and ADF content, and temperature, and positively correlated with pasture protein and HWSC content, and rainfall.

Figure 6.7. Correlation matrix of bacterial taxa, macro nutrients of pasture and climate variables.



Legend: Each square on the matrix represents the degree of correlation between two corresponding variables, and is illustrated by the colour intensity and size of the square, as shown on the scale below the matrix; i.e. small light blue to large dark blue squares represent positive correlation from >0 to +1; small light red to large dark red squares represent negative correlation from <0 to -1. The bacterial taxa included are as follows: Genus1 –Unclassified genus within family Ruminococcaceae; Genus2 – Unclassified genus within order Bacteroidales; Genus3 – Unclassified genus within family Lachnospiraceae; Genus4 – Unclassified genus within order Clostridiales. DM – Dry matter (%); GE – Gross energy (KJ/g); NDF – Neutral detergent fibre (%); ADF – Acid detergent fibre (%); NSC – Non-structural carbohydrates (%); HWSC – Hot water soluble carbohydrates (%); Rainfall (mm) and Temperature (°C).

6.5 Discussion

The present study investigated the effects of seasonal changes in the nutrient composition of pasture on the diversity of faecal microbiota in horses grazing on typical New Zealand pasture. Currently, there are limited data on the hindgut or faecal microbiota profile of pasture-fed horses, and this study is the second (after our previous work (Fernandes *et al.*, 2014)) to examine the effects of dietary change on the diversity of faecal microbiota in pasture-fed horses. The results of this study showed significant effects of diet, horse, and season on the diversity within the faecal bacterial community, which was dominated by four genera within two phyla – Firmicutes and Bacteroidetes. The faecal bacterial community profile identified in this study was similar to the profile reported in our previous work on pasture-fed horses in New Zealand [6].

The faecal samples collected and bacterial sequences obtained in the present study were representative of the year-round population of faecal microbiota, which comprised a rich and diverse bacterial community. The dominant bacterial phyla detected in the present study were consistent with reports from previous studies that used the Illumina next-generation sequencing technique to examine caecal and faecal microbiota in horses (Kristoffersen, 2014, Moreau *et al.*, 2014, Costa *et al.*, 2015c). Some of the bacterial phyla detected were similar to those identified in other studies of horses using various molecular techniques, but the relative abundances reported in those studies varied considerably (Kobayashi *et al.*, 2006, Costa *et al.*, 2012, Dougal *et al.*, 2013, O' Donnell *et al.*, 2013). This was perhaps due to the variation in the type of horse selected, the type and composition of the diet fed to the horses, the geographical location, inconsistencies in the management practices, and the variation in molecular and bioinformatics analyses used across the studies (Costa and Weese, 2012). Although similarities in results were apparent, caution is warranted in comparing some of the conflicting findings reported in previous studies to the results obtained in the present study.

Kobayashi and co-workers (Kobayashi *et al.*, 2006) were the first to report a seasonal variation in the diversity of faecal microbiota in horses fed summer versus winter pasture, using conventional microscopy to enumerate the population of the microbiota. However, given that the faecal bacterial community in forage-fed horses is diet specific (Fernandes *et al.*, 2014), the underlying differences in diet (grassland pasture comprising of timothy grass offered during summer and woodland pasture comprising of bamboo grass offered during

winter), and perhaps its nutrient composition, may have confounded the effect of season on the microbial diversity reported in that study (Kobayashi *et al.*, 2006).

The significant effects of diet reported in the present study, were in agreement with the findings of our previous work in New Zealand Thoroughbred horses that were also fed forage-only diets (Fernandes *et al.*, 2014). The bacterial diversity was higher when horses were fed exclusively on pasture in both studies, when compared to the period when pasture was supplemented with hay or when the horses were fed on a chopped ensiled forage. While there was a temporal effect on the diversity of faecal bacteria within the two diet periods in the present study, the supplementation of hay appeared to dampen the effects of variations in pasture composition. This dampening effect could be associated with the relatively consistent nutrient composition of hay usually harvested at a fixed time during the year, when compared to the variation observed in pasture grazed over several months. However, again caution is warranted in extrapolating this finding to other sources and types of hay, because there may be substantial variation in the nutrient composition depending on the type of grass/legume, stage of growth, climate factors, time of harvest, method of processing and storage, which may vary considerably between batches of hay.

Furthermore, Table 6.1 in the present study showed decreases in %CP, %NSC, %HWSC and DE of hay that was fed over five months after open/barn storage, even though the hay was harvested and processed as a single batch. This finding indicates that storage conditions have a negative effect on the nutritive value of hay, and perhaps, this may be true for other fermented forages preserved under field conditions over extended periods (Collins *et al.*, 1995, Turner *et al.*, 2002). However, a shelf-life study conducted on a batch of chopped ensiled forage (prepared commercially using forages harvested as a single batch, processed via a controlled fermentation technique and stored anaerobically in polythene-wrapped packaging) showed no change in nutritive value over a storage period of 12 months (Patel, 2014). This type of an ensiled forage appears to have a more stable nutrient composition when compared to hay or pasture (present study), and also supports a high diversity of faecal microbiota similar to horses grazing on pasture (Fernandes *et al.*, 2014).

The variation in pasture composition and the fluctuations in diversity of faecal microbiota observed in the present study may have implications for grazing management and the preparation of conserved forages for horses susceptible to perturbations of the hindgut microbiota. It may be hypothesised that good quality hay or ensiled forages of a similar

nutrient composition could support a relatively consistent population of faecal microbiota by minimising the fluctuations in microbial diversity observed in horses when fed on pasture. Further investigations on the effects of different types of conserved forages on the diversity of the faecal microbiota in horses fed over an extended period are required.

The temporal effects of season and the more subtle effect of month on the diversity of the bacterial community in faeces reported in the present study, appeared to be driven by the variation in the nutrient composition of pasture. When the pasture was growing (vegetative phase, high %CP and low %CHO), Firmicutes dominated the bacterial community, whereas when the pasture was dry (drought stressed, low %CP and high %CHO), the abundance of Bacteroidetes increased, at the expense of Firmicutes. In particular, the trend for increasing DM content during the months from January to March (drought period), was associated with low pasture growth, which significantly affected the nutrient composition. This spike observed in the %DM was in agreement with previous reports from the region (Litherland *et al.*, 2002, Hirst, 2011), and was closely associated with climatic factors (low rainfall and high temperature). The minimal variation in HWSCs reported in the present study may have been biased by the time at which the pasture samples were collected (around mid-day), when the HWSC content is potentially at its lowest in comparison to the early-morning or late-afternoon hours (Longland *et al.*, 1999, Longland and Byrd, 2006). Subtle differences were observed in the bacterial diversity between the months when horses were grazing exclusively on pasture (April, May – winter/limited pasture; November, December – spring/lush pasture and January, February, March – summer/dry pasture) or when limited pasture was supplemented with hay (June, July, August, September, October). This finding was unique and provided evidence in support of the sensitivity of the next-generation sequencing technique to identify these small changes in the diversity of faecal microbial populations (Costa *et al.*, 2015c).

While the main findings of the present study supported the hypothesis that pasture composition influences the diversity of the faecal microbiota of horses, variation was observed between horses within diet periods. Nevertheless, only a few less abundant phyla differed between horses, whereas the most dominant phyla (Firmicutes and Bacteroidetes) were not significantly different. This finding indicated that the group of horses in the study shared a core microbiome, with minimal variation between the horses, and is similar to previous reports on other groups of horses where the diet and management was relatively consistent (Dougal *et al.*, 2013, O' Donnell *et al.*, 2013, Dougal *et al.*, 2014). However, the subtle differences in the less abundant bacterial taxa detected between individual horses, even though they were

managed as a single cohort under similar conditions, may be associated with the feed intake of individual horses and the retention times of digesta through the gastrointestinal tract. Some horses consume more feed than others, a behaviour that appears to affect the transit time of digesta through the gastrointestinal tract (Pearson and Merritt, 1991, Pearson *et al.*, 2006), and consequently, affects the time available for microbial digestion (Fernandes *et al.*, 2016, submitted). This digestive strategy may explain one of the reasons why some horses (due to their inherent feeding behaviour and individual hindgut microbiota) may adapt to dietary changes better than other horses or why some horses may be predisposed to hindgut dysbiosis that induces acute colic or laminitis (Moreau *et al.*, 2014, Weese *et al.*, 2015). This further suggests that the population of hindgut/faecal microbiota in some horses is more resilient than in other horses, i.e. the microbial populations of some horses bounce back or adapt better than other horses, although this requires further investigation.

When the %DM of pasture increased, the relative abundance of the phylum Bacteroidetes increased and that of Firmicutes decreased, whilst the remaining phyla of lower relative abundances remained fairly constant. This finding suggests that major shifts in bacterial diversity and abundance can occur in these two phyla, and they should be considered important when monitoring changes in microbial populations. Similar shifts in the Firmicutes:Bacteroidetes ratio have been reported in other studies on the gut microbiome of horses (Costa *et al.*, 2012, Faubladier *et al.*, 2013, Dougal *et al.*, 2014, Costa *et al.*, 2015c) and humans (Mariat *et al.*, 2009, Turnbaugh *et al.*, 2009, De Filippo *et al.*, 2010, Yatsunenko *et al.*, 2012) in relation to dietary manipulation. Furthermore, within the phylum Firmicutes, three of the most dominant genera were classified within the order Clostridiales, indicating the importance of this group of bacteria and its association with dietary changes and development of intestinal dysbiosis (Weese *et al.*, 2015). However, the functional roles of these unclassified genera within the order Clostridiales are still unknown and require further investigation.

6.6 Conclusion

The present study showed that the faecal microbiota of pasture-fed horses was highly diverse, and their community structure was diet-specific and associated with the macronutrient composition of the forage. The population structure of the bacterial community, albeit dominated by two bacterial phyla, was dynamic with relative abundances of bacterial genera that fluctuated over time. These fluctuations appeared to be driven by seasonal changes in

pasture composition associated in turn with climate factors such as rainfall and temperature. It would be interesting to investigate the resilience of faecal microbiota following dietary alterations, i.e. how do their abundances bounce back after a shift during the dry pasture periods? However, measuring this aspect was beyond the scope of the present study, which only collected snapshot data at monthly intervals for a 12-month period. The outcome of this study suggests future studies can be designed to examine the rate of change in the diversity of faecal microbiota and its resilience following dietary alterations.

6.7 Acknowledgments

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Appendix D

This appendix provides supplementary information on the tables and figures cited in Chapter 6.

D-1. Text S1. Detailed materials and methods.

D-2. Table S1. Metadata on the faecal samples (n=118) included in the study on the seasonal variation in the faecal microbiota of pasture-fed horses (n=10).

D-3. Table S2. Comparison of mean relative abundance at the phylum level between diet periods.

D-4. Table S3. Comparison of the mean relative abundance at the genus level between diet periods.

D-5. Table S4. Comparison of mean relative abundance at the phylum level between horses.

D-6. Table S5. Comparison of the mean relative abundance at the genus level between horses.

D-7. Figure S1. Principal coordinate analysis of the faecal bacterial community by season and month.

D-8. Figure S2. Season-wise hierarchial clustering of the faecal bacterial community of horses included in the study.

D-9. An illustration of the seasonal variation in pasture during the study period.

D-10. Table S6. Comparison of mean relative abundance at the phylum level between seasons.

D-11. Table S7. Comparison of mean relative abundance at the genus level between seasons.

D-12. Table S8. Comparison of mean relative abundance at the phylum level between months.

D-13. Table S9. Comparison of mean relative abundance at the genus level between months.

D-1. Text S1. Detailed materials and methods.

DNA extraction method

The cells were disrupted by bead-beating for 4 min at 2100 oscillations s^{-1} (Mini-Beadbeater-96, BioSpec, Bartlesville, OK, USA), with 0.7 g Zirconia beads (0.1 mm; dnature, Gisborne, New Zealand), 550 µl phenol-chloroform-isoamylalcohol (25:24:1 vol:vol:vol; pH 8), 282 µl buffer A (200 mMNaCl, 200 mMTria, 20 mM EDTA, pH 8 with NaOH), 268 µl PM buffer (Qiagen, Hilden, Germany), and 200 µl sodium dodecyl sulphate (20% wt/vol) (Rius *et al.*, 2012, Kittelmann *et al.*, 2014). After centrifugation at 20,000 x *g* at 4°C for 20 min, 350 µl of the supernatant was removed, and treated at 37°C for 15 min with 5 µl RNase A (Life Technologies, Thermo Fisher Scientific, Auckland, New Zealand) (Healey *et al.*, 2014). The supernatant was then mixed with 650 µl PM buffer (Qiagen) and processed through the QIAquick column by applying a vacuum with a QIAvac 96 vacuum manifold (Qiagen). The membrane-bound DNA was washed with 750 µl PE buffer (Qiagen) twice as recommended by the manufacturer, and DNA was eluted in 80 µl elution buffer (10 mMTris; pH 8.5 with HCl) and stored at -80°C.

PCR amplification and sequencing protocol

Primer and adapter sequences

To amplify and sequence the V3-V4 hypervariable region of the 16S rRNA gene, specific bacterial primer sequences were selected, S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth *et al.*, 2012). The full length primer sequences, using standard IUPAC nucleotide nomenclature, were as follows:

16S Amplicon PCR Forward Primer:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’

16S Amplicon PCR Reverse Primer:

5'-
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC
C-3’

The Illumina overhang adapter sequences added to the locus-specific sequences were:

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]

Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]

Amplicon PCR

The amplicons were prepared using 2.5 µlgDNA (5 ng/ µl), 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 12.5 µl 2x KAPA HiFiHotStart Ready Mix, to make a master mix reaction of 25 µl. PCR was performed on a Thermocycler ProS (Eppendorf, Hamburg, Germany) using the following program: initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds, followed by a final elongation at 72°C for 5 minutes and held at 4°C. The amplicons were then purified using AMPure XP beads (AMPure, Agencourt, Beckman Coulter, Beverly, MA, USA).

Index PCR

The linker primer sequence used for the samples was 5'-GTGCCAGCMGCCGCGGTAA-3' and the 8 bp unique barcode pairs are given in Table S1. The dual indices and Illumina sequencing adapters were attached to the PCR amplicons by the following protocol: 5 µlgDNA, 5 µlNextera XT Index Primer 1 (N7xx), 5 µlNextera XT Index Primer 2 (S5xx), 25 µl 2x KAPA HiFiHotStart Ready Mix, 10 µl PCR grade water, to make a total master mix of 50 µl. PCR was performed on a Thermocycler ProS (Eppendorf) using the following program: initial denaturation at 95°C for 3 minutes, 8 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds, followed by a final elongation at 72°C for 5 minutes and held at 4°C. The amplicons were then purified using AMPure XP beads (AMPure) (Fadrosh *et al.*, 2014).

Library validation

A sample of 1 µl of a 1:50 dilution of the final library was run on a Bioanalyzer DNA 1000 chip (2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to verify the size of the amplicons, which was expected to be ~630 bp when using the V3 and V4 primer pairs in the protocol.

D-2. Table S1. Metadata on the faecal samples (n=118) included in the study on the seasonal variation in the faecal microbiota of pasture-fed horses (n=10).

SampleID	BarcodeSequence1	BarcodeSequence2	HorseID	Breed	Age	Month	Season	Diet	Location
Jan_H1	TAAGGCGA	TAGATCGC	H1	Standardbred	26	January	Summer	Pasture	Paddock_3
Jan_H2	CGTACTAG	TAGATCGC	H2	Thoroughbred	15	January	Summer	Pasture	Paddock_3
Jan_H3	AGGCAGAA	TAGATCGC	H3	Standardbred	9	January	Summer	Pasture	Paddock_3
Jan_H4	TCCTGAGC	TAGATCGC	H4	Standardbred	19	January	Summer	Pasture	Paddock_3
Jan_H5	GGACTCCT	TAGATCGC	H5	Thoroughbred	12	January	Summer	Pasture	Paddock_3
Jan_H6	TAGGCATG	TAGATCGC	H6	Standardbred	17	January	Summer	Pasture	Paddock_3
Jan_H7	CTCTCTAC	TAGATCGC	H7	Standardbred	8	January	Summer	Pasture	Paddock_3
Jan_H8	CAGAGAGG	TAGATCGC	H8	Standardbred	12	January	Summer	Pasture	Paddock_3
Jan_H9	GCTACGCT	TAGATCGC	H9	Standardbred	19	January	Summer	Pasture	Paddock_3
Jan_H10	CGAGGCTG	TAGATCGC	H10	Standardbred	14	January	Summer	Pasture	Paddock_3
Feb_H1	AAGAGGCA	TAGATCGC	H1	Standardbred	26	February	Summer	Pasture	Paddock_3
Feb_H2	GTAGAGGA	TAGATCGC	H2	Thoroughbred	15	February	Summer	Pasture	Paddock_3
Feb_H3	TAAGGCGA	CTCTCTAT	H3	Standardbred	9	February	Summer	Pasture	Paddock_3
Feb_H4	CGTACTAG	CTCTCTAT	H4	Standardbred	19	February	Summer	Pasture	Paddock_3
Feb_H5	AGGCAGAA	CTCTCTAT	H5	Thoroughbred	12	February	Summer	Pasture	Paddock_3
Feb_H6	TCCTGAGC	CTCTCTAT	H6	Standardbred	17	February	Summer	Pasture	Paddock_3
Feb_H7	GGACTCCT	CTCTCTAT	H7	Standardbred	8	February	Summer	Pasture	Paddock_3
Feb_H8	TAGGCATG	CTCTCTAT	H8	Standardbred	12	February	Summer	Pasture	Paddock_3
Feb_H9	CTCTCTAC	CTCTCTAT	H9	Standardbred	19	February	Summer	Pasture	Paddock_3
Feb_H10	CAGAGAGG	CTCTCTAT	H10	Standardbred	14	February	Summer	Pasture	Paddock_3
Mar_H1	GCTACGCT	CTCTCTAT	H1	Standardbred	26	March	Autumn	Pasture	Paddock_3
Mar_H2	CGAGGCTG	CTCTCTAT	H2	Thoroughbred	15	March	Autumn	Pasture	Paddock_3
Mar_H3	AAGAGGCA	CTCTCTAT	H3	Standardbred	9	March	Autumn	Pasture	Paddock_3
Mar_H4	GTAGAGGA	CTCTCTAT	H4	Standardbred	19	March	Autumn	Pasture	Paddock_3
Mar_H5	TAAGGCGA	TATCCTCT	H5	Thoroughbred	12	March	Autumn	Pasture	Paddock_3
Mar_H6	CGTACTAG	TATCCTCT	H6	Standardbred	17	March	Autumn	Pasture	Paddock_3

Mar_H7	AGGCAGAA	TATCCTCT	H7	Standardbred	8	March	Autumn	Pasture	Paddock_3
Mar_H8	TCCTGAGC	TATCCTCT	H8	Standardbred	12	March	Autumn	Pasture	Paddock_3
Mar_H9	GGACTCCT	TATCCTCT	H9	Standardbred	19	March	Autumn	Pasture	Paddock_3
Mar_H10	TAGGCATG	TATCCTCT	H10	Standardbred	14	March	Autumn	Pasture	Paddock_3
Apr_H1	CTCTCTAC	TATCCTCT	H1	Standardbred	26	April	Autumn	Pasture	Paddock_1
Apr_H2	CAGAGAGG	TATCCTCT	H2	Thoroughbred	15	April	Autumn	Pasture	Paddock_1
Apr_H3	GCTACGCT	TATCCTCT	H3	Standardbred	9	April	Autumn	Pasture	Paddock_1
Apr_H4	CGAGGCTG	TATCCTCT	H4	Standardbred	19	April	Autumn	Pasture	Paddock_1
Apr_H5	AAGAGGCA	TATCCTCT	H5	Thoroughbred	12	April	Autumn	Pasture	Paddock_1
Apr_H6	GTAGAGGA	TATCCTCT	H6	Standardbred	17	April	Autumn	Pasture	Paddock_1
Apr_H7	TAAGGCGA	AGAGTAGA	H7	Standardbred	8	April	Autumn	Pasture	Paddock_1
Apr_H8	CGTACTAG	AGAGTAGA	H8	Standardbred	12	April	Autumn	Pasture	Paddock_1
Apr_H9	AGGCAGAA	AGAGTAGA	H9	Standardbred	19	April	Autumn	Pasture	Paddock_1
Apr_H10	TCCTGAGC	AGAGTAGA	H10	Standardbred	14	April	Autumn	Pasture	Paddock_1
May_H1	GGACTCCT	AGAGTAGA	H1	Standardbred	26	May	Autumn	Pasture	Paddock_1
May_H2	TAGGCATG	AGAGTAGA	H2	Thoroughbred	15	May	Autumn	Pasture	Paddock_1
May_H3	CTCTCTAC	AGAGTAGA	H3	Standardbred	9	May	Autumn	Pasture	Paddock_1
May_H4	CAGAGAGG	AGAGTAGA	H4	Standardbred	19	May	Autumn	Pasture	Paddock_1
May_H5	GCTACGCT	AGAGTAGA	H5	Thoroughbred	12	May	Autumn	Pasture	Paddock_1
May_H6	CGAGGCTG	AGAGTAGA	H6	Standardbred	17	May	Autumn	Pasture	Paddock_1
May_H7	AAGAGGCA	AGAGTAGA	H7	Standardbred	8	May	Autumn	Pasture	Paddock_1
May_H8	GTAGAGGA	AGAGTAGA	H8	Standardbred	12	May	Autumn	Pasture	Paddock_1
May_H10	CGTACTAG	GTAAGGAG	H10	Standardbred	14	May	Autumn	Pasture	Paddock_1
Jun_H1	AGGCAGAA	GTAAGGAG	H1	Standardbred	26	June	Winter	Pasture+Hay	Paddock_1
Jun_H2	TCCTGAGC	GTAAGGAG	H2	Thoroughbred	15	June	Winter	Pasture+Hay	Paddock_1
Jun_H3	GGACTCCT	GTAAGGAG	H3	Standardbred	9	June	Winter	Pasture+Hay	Paddock_1
Jun_H4	TAGGCATG	GTAAGGAG	H4	Standardbred	19	June	Winter	Pasture+Hay	Paddock_1
Jun_H5	CTCTCTAC	GTAAGGAG	H5	Thoroughbred	12	June	Winter	Pasture+Hay	Paddock_1
Jun_H6	CAGAGAGG	GTAAGGAG	H6	Standardbred	17	June	Winter	Pasture+Hay	Paddock_1

Jun_H7	GCTACGCT	GTAAGGAG	H7	Standardbred	8	June	Winter	Pasture+Hay	Paddock_1
Jun_H8	CGAGGCTG	GTAAGGAG	H8	Standardbred	12	June	Winter	Pasture+Hay	Paddock_1
Jun_H9	AAGAGGCA	GTAAGGAG	H9	Standardbred	19	June	Winter	Pasture+Hay	Paddock_1
Jun_H10	GTAGAGGA	GTAAGGAG	H10	Standardbred	14	June	Winter	Pasture+Hay	Paddock_1
Jul_H1	TAAGGCGA	ACTGCATA	H1	Standardbred	26	July	Winter	Pasture+Hay	Paddock_1
Jul_H2	CGTACTAG	ACTGCATA	H2	Thoroughbred	15	July	Winter	Pasture+Hay	Paddock_1
Jul_H3	AGGCAGAA	ACTGCATA	H3	Standardbred	9	July	Winter	Pasture+Hay	Paddock_1
Jul_H4	TCCTGAGC	ACTGCATA	H4	Standardbred	19	July	Winter	Pasture+Hay	Paddock_1
Jul_H5	GGACTCCT	ACTGCATA	H5	Thoroughbred	12	July	Winter	Pasture+Hay	Paddock_1
Jul_H6	TAGGCATG	ACTGCATA	H6	Standardbred	17	July	Winter	Pasture+Hay	Paddock_1
Jul_H7	CTCTCTAC	ACTGCATA	H7	Standardbred	8	July	Winter	Pasture+Hay	Paddock_1
Jul_H8	CAGAGAGG	ACTGCATA	H8	Standardbred	12	July	Winter	Pasture+Hay	Paddock_1
Jul_H9	GCTACGCT	ACTGCATA	H9	Standardbred	19	July	Winter	Pasture+Hay	Paddock_1
Jul_H10	CGAGGCTG	ACTGCATA	H10	Standardbred	14	July	Winter	Pasture+Hay	Paddock_1
Aug_H1	AAGAGGCA	ACTGCATA	H1	Standardbred	26	August	Winter	Pasture+Hay	Paddock_1
Aug_H2	GTAGAGGA	ACTGCATA	H2	Thoroughbred	15	August	Winter	Pasture+Hay	Paddock_1
Aug_H3	TAAGGCGA	AAGGAGTA	H3	Standardbred	9	August	Winter	Pasture+Hay	Paddock_1
Aug_H4	CGTACTAG	AAGGAGTA	H4	Standardbred	19	August	Winter	Pasture+Hay	Paddock_1
Aug_H5	AGGCAGAA	AAGGAGTA	H5	Thoroughbred	12	August	Winter	Pasture+Hay	Paddock_1
Aug_H7	GGACTCCT	AAGGAGTA	H7	Standardbred	8	August	Winter	Pasture+Hay	Paddock_1
Aug_H8	TAGGCATG	AAGGAGTA	H8	Standardbred	12	August	Winter	Pasture+Hay	Paddock_1
Aug_H9	CTCTCTAC	AAGGAGTA	H9	Standardbred	19	August	Winter	Pasture+Hay	Paddock_1
Aug_H10	CAGAGAGG	AAGGAGTA	H10	Standardbred	14	August	Winter	Pasture+Hay	Paddock_1
Sept_H1	GCTACGCT	AAGGAGTA	H1	Standardbred	26	September	Spring	Pasture+Hay	Paddock_1
Sept_H2	CGAGGCTG	AAGGAGTA	H2	Thoroughbred	15	September	Spring	Pasture+Hay	Paddock_1
Sept_H3	AAGAGGCA	AAGGAGTA	H3	Standardbred	9	September	Spring	Pasture+Hay	Paddock_1
Sept_H4	GTAGAGGA	AAGGAGTA	H4	Standardbred	19	September	Spring	Pasture+Hay	Paddock_1
Sept_H5	TAAGGCGA	CTAAGCCT	H5	Thoroughbred	12	September	Spring	Pasture+Hay	Paddock_1
Sept_H6	CGTACTAG	CTAAGCCT	H6	Standardbred	17	September	Spring	Pasture+Hay	Paddock_1

Sept_H7	AGGCAGAA	CTAAGCCT	H7	Standardbred	8	September	Spring	Pasture+Hay	Paddock_1
Sept_H8	TCCTGAGC	CTAAGCCT	H8	Standardbred	12	September	Spring	Pasture+Hay	Paddock_1
Sept_H9	GGACTCCT	CTAAGCCT	H9	Standardbred	19	September	Spring	Pasture+Hay	Paddock_1
Sept_H10	TAGGCATG	CTAAGCCT	H10	Standardbred	14	September	Spring	Pasture+Hay	Paddock_1
Oct_H1	CTCTCTAC	CTAAGCCT	H1	Standardbred	26	October	Spring	Pasture+Hay	Paddock_1
Oct_H2	CAGAGAGG	CTAAGCCT	H2	Thoroughbred	15	October	Spring	Pasture+Hay	Paddock_1
Oct_H3	GCTACGCT	CTAAGCCT	H3	Standardbred	9	October	Spring	Pasture+Hay	Paddock_1
Oct_H4	CGAGGCTG	CTAAGCCT	H4	Standardbred	19	October	Spring	Pasture+Hay	Paddock_1
Oct_H5	AAGAGGCA	CTAAGCCT	H5	Thoroughbred	12	October	Spring	Pasture+Hay	Paddock_1
Oct_H6	GTAGAGGA	CTAAGCCT	H6	Standardbred	17	October	Spring	Pasture+Hay	Paddock_1
Oct_H7	TAAGGCGA	GCGTAAGA	H7	Standardbred	8	October	Spring	Pasture+Hay	Paddock_1
Oct_H8	CGTACTAG	GCGTAAGA	H8	Standardbred	12	October	Spring	Pasture+Hay	Paddock_1
Oct_H9	AGGCAGAA	GCGTAAGA	H9	Standardbred	19	October	Spring	Pasture+Hay	Paddock_1
Oct_H10	TCCTGAGC	GCGTAAGA	H10	Standardbred	14	October	Spring	Pasture+Hay	Paddock_1
Nov_H1	GGACTCCT	GCGTAAGA	H1	Standardbred	26	November	Spring	Pasture	Paddock_1
Nov_H2	TAGGCATG	GCGTAAGA	H2	Thoroughbred	15	November	Spring	Pasture	Paddock_1
Nov_H3	CTCTCTAC	GCGTAAGA	H3	Standardbred	9	November	Spring	Pasture	Paddock_1
Nov_H4	CAGAGAGG	GCGTAAGA	H4	Standardbred	19	November	Spring	Pasture	Paddock_1
Nov_H5	GCTACGCT	GCGTAAGA	H5	Thoroughbred	12	November	Spring	Pasture	Paddock_1
Nov_H6	CGAGGCTG	GCGTAAGA	H6	Standardbred	17	November	Spring	Pasture	Paddock_1
Nov_H7	AAGAGGCA	GCGTAAGA	H7	Standardbred	8	November	Spring	Pasture	Paddock_1
Nov_H8	GTAGAGGA	GCGTAAGA	H8	Standardbred	12	November	Spring	Pasture	Paddock_1
Nov_H9	TAAGGCGA	CTCTCTAT	H9	Standardbred	19	November	Spring	Pasture	Paddock_1
Nov_H10	CGTACTAG	CTCTCTAT	H10	Standardbred	14	November	Spring	Pasture	Paddock_1
Dec_H1	AGGCAGAA	CTCTCTAT	H1	Standardbred	26	December	Summer	Pasture	Paddock_2
Dec_H2	TCCTGAGC	CTCTCTAT	H2	Thoroughbred	15	December	Summer	Pasture	Paddock_2
Dec_H3	GGACTCCT	CTCTCTAT	H3	Standardbred	9	December	Summer	Pasture	Paddock_2
Dec_H4	TAGGCATG	CTCTCTAT	H4	Standardbred	19	December	Summer	Pasture	Paddock_2
Dec_H5	CTCTCTAC	CTCTCTAT	H5	Thoroughbred	12	December	Summer	Pasture	Paddock_2

Dec_H6	CAGAGAGG	CTCTCTAT	H6	Standardbred	17	December	Summer	Pasture	Paddock_2
Dec_H7	GCTACGCT	CTCTCTAT	H7	Standardbred	8	December	Summer	Pasture	Paddock_2
Dec_H8	CGAGGCTG	CTCTCTAT	H8	Standardbred	12	December	Summer	Pasture	Paddock_2
Dec_H9	AAGAGGCA	CTCTCTAT	H9	Standardbred	19	December	Summer	Pasture	Paddock_2
Dec_H10	GTAGAGGA	CTCTCTAT	H10	Standardbred	14	December	Summer	Pasture	Paddock_2

D-3. Table S2. Comparison of mean relative abundance at the phylum level between diet periods.

Taxon	Pasture	Pasture + Hay	P value
Firmicutes	0.611	0.680	<0.001*
Bacteroidetes	0.237	0.194	0.002*
Verrucomicrobia	0.039	0.033	0.003*
Unassigned phyla	0.033	0.023	<0.001*
Actinobacteria	0.018	0.015	0.024
TM7	0.017	0.017	0.737
Spirochaetes	0.016	0.010	0.002*
Proteobacteria	0.006	0.005	<0.001*
Tenericutes	0.006	0.005	0.178
Cyanobacteria	0.004	0.003	0.003*
Fibrobacteres	0.003	0.003	0.591
Euryarchaeota	0.002	0.001	0.005
WPS-2	0.001	0.002	0.119
Fusobacteria	<0.001	<0.001	0.745
Other Phyla <1%	0.007	0.007	0.336

*Level of significance was P≤0.003 after Bonferroni correction for multiple comparisons

D-4. Table S3. Comparison of the mean relative abundance at the genus level between diet periods.

Taxon	Pasture	Pasture+Hay	P value
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>unclassified genus	0.206	0.234	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.135	0.136	0.581
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.116	0.140	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>unclassified family>unclassified genus	0.108	0.086	0.002
Unassigned genera	0.033	0.023	<0.001*
Verrucomicrobia>Verruco-5>WCHB1-41>RFP12>unclassified genus	0.023	0.019	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>Prevotellaceae>Prevotella	0.023	0.020	0.132
Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>unclassified genus	0.021	0.024	0.042
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Ruminococcus	0.021	0.023	0.366
Bacteroidetes>Bacteroidia>Bacteroidales>BS11>unclassified genus	0.019	0.021	0.920
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>unclassified genus	0.018	0.020	0.876
TM7>TM7-3>CW040>F16>unclassified genus	0.017	0.017	0.702
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>YRC22	0.015	0.015	0.668
Verrucomicrobia>Verrucomicrobiae>Verrucomicrobiales>Verrucomicrobiaceae>Akkermansia	0.014	0.014	0.058
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>CF231	0.013	0.007	<0.001*
Spirochaetes>Spirochaetes>Spirochaetales>Spirochaetaceae>Treponema	0.012	0.009	0.011
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Coprococcus	0.011	0.013	0.046
Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>p-75-a5	0.011	0.012	0.310
Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Phascolarctobacterium	0.009	0.006	0.005
Bacteroidetes>Bacteroidia>Bacteroidales>RF16>unclassified genus	0.008	0.003	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>BF311	0.008	0.007	0.036
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Oscillospira	0.008	0.009	0.307
Bacteroidetes>Bacteroidia>Bacteroidales>Porphyromonadaceae>Paludibacter	0.008	0.002	0.036
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Clostridium	0.007	0.008	0.544

Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	0.007	0.013	<0.001*
Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>unclassified genus	0.007	0.008	0.063
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae;Other	0.006	0.005	0.036
Firmicutes>Clostridia>Clostridiales>Christensenellaceae>unclassified genus	0.006	0.008	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>[Prevotella]	0.005	0.004	0.019
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Blautia	0.005	0.004	0.288
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Pseudobutyrivibrio	0.004	0.004	0.221
Firmicutes>Clostridia>Clostridiales>Eubacteriaceae>Pseudoramibacter_Eubacterium	0.004	0.005	0.881
Firmicutes>Bacilli>Lactobacillales>Lactobacillaceae>Lactobacillus	0.004	0.008	<0.001*
Tenericutes>Mollicutes>RF39>unclassified family>unclassified genus	0.004	0.005	0.387
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>Bacteroides	0.004	0.004	0.003
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.004	0.003	0.044
Cyanobacteria>4C0d-2>YS2>unclassified family>unclassified genus	0.004	0.003	0.004
Fibrobacteres>Fibrobacteria>Fibrobacterales>Fibrobacteraceae>Fibrobacter	0.003	0.003	0.591
Spirochaetes>MVP-15>PL-11B10>unclassified family>unclassified genus	0.003	0.001	0.864
Actinobacteria>Actinobacteria>Actinomycetales>Nocardiaceae>Rhodococcus	0.002	0.001	<0.001*
Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>RFN20	0.002	0.001	0.031
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>unclassified genus	0.002	0.002	0.128
Firmicutes>Clostridia>Clostridiales>Veillonellaceae>unclassified genus	0.002	0.004	0.022
WPS-2>unclassified class>unclassified order>unclassified family>unclassified genus	0.001	0.002	0.119
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Sarcina	0.001	0.003	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>[Ruminococcus]	0.001	0.003	0.484
Euryarchaeota>Methanobacteria>Methanobacteriales>Methanobacteriaceae>Methanobrevibacter	0.001	0.001	0.741
Verrucomicrobia>Verruco-5>LD1-PB3>unclassified family>unclassified genus	<0.001	<0.001	0.549
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Epulopiscium	<0.001	0.001	0.015
Proteobacteria>Gammaproteobacteria>Enterobacteriales>Enterobacteriaceae>unclassified genus	<0.001	<0.001	0.049

Fusobacteria>Fusobacteriia>Fusobacteriales>Fusobacteriaceae>Fusobacterium	<0.001	<0.001	0.545
Other Genera <1%	0.049	0.039	<0.001*

*Level of significance was P≤0.001 after Bonferroni correction for multiple comparisons

D-5. Table S4. Comparison of mean relative abundance at the phylum level between horses.

Taxon	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	P value
Firmicutes	0.608	0.655	0.605	0.635	0.634	0.656	0.648	0.655	0.620	0.680	0.281
Bacteroidetes	0.239	0.224	0.24	0.225	0.231	0.195	0.221	0.203	0.232	0.178	0.436
Unassigned phyla	0.033	0.028	0.032	0.027	0.033	0.026	0.025	0.026	0.029	0.030	0.199
Verrucomicrobia	0.032	0.027	0.036	0.040	0.029	0.04	0.027	0.04	0.045	0.053	0.001*
TM7	0.025	0.009	0.024	0.019	0.017	0.018	0.015	0.022	0.01	0.012	0.007
Spirochaetes	0.017	0.018	0.015	0.011	0.014	0.010	0.015	0.011	0.017	0.009	0.049
Actinobacteria	0.016	0.014	0.017	0.015	0.015	0.023	0.020	0.016	0.018	0.017	0.359
Tenericutes	0.006	0.005	0.008	0.006	0.005	0.006	0.006	0.006	0.005	0.004	0.002*
Proteobacteria	0.005	0.005	0.005	0.005	0.005	0.009	0.006	0.006	0.006	0.004	0.180
Cyanobacteria	0.005	0.004	0.004	0.003	0.004	0.003	0.003	0.004	0.003	0.002	0.009
Fibrobacteres	0.004	0.003	0.004	0.003	0.005	0.003	0.003	0.001	0.004	0.002	0.098
Euryarchaeota	0.002	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.088
WPS-2	0.002	0.001	0.002	0.001	0.001	0.004	0.002	0.002	0.002	0.001	0.049
Fusobacteria	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.826
Other Phyla <1%	0.007	0.005	0.007	0.008	0.007	0.006	0.006	0.007	0.01	0.008	0.001*

*Level of significance was P≤0.003 after Bonferroni correction for multiple comparisons

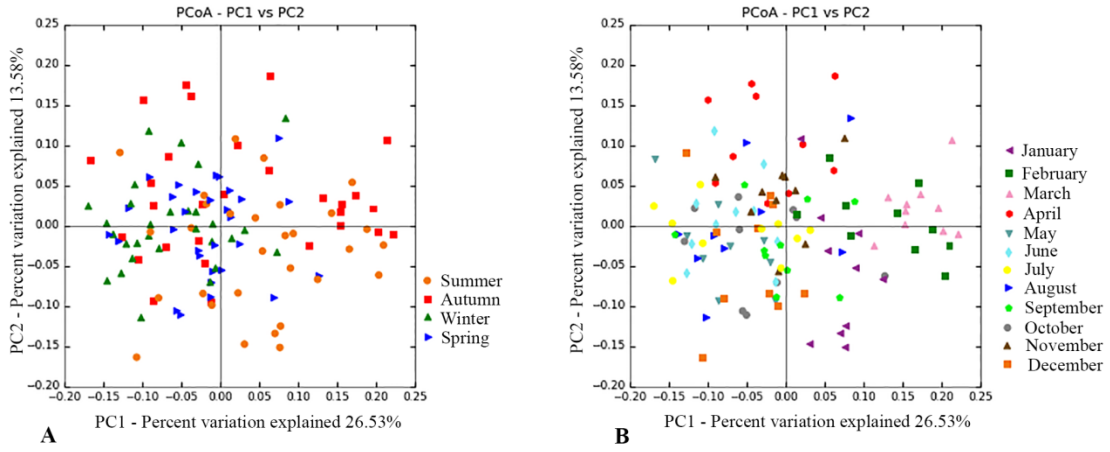
D-6. Table S5. Comparison of the mean relative abundance at the genus level between horses.

Taxon	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	P value
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>unclassified genus	0.203	0.221	0.218	0.210	0.223	0.221	0.220	0.243	0.207	0.208	0.450
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.140	0.163	0.104	0.141	0.122	0.145	0.127	0.120	0.140	0.153	0.002
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.119	0.116	0.128	0.125	0.129	0.129	0.127	0.129	0.117	0.14	0.737
Bacteroidetes>Bacteroidia>Bacteroidales>unclassified family>unclassified genus	0.114	0.094	0.110	0.098	0.105	0.091	0.109	0.084	0.110	0.077	0.259
Unassigned genera	0.033	0.028	0.032	0.027	0.033	0.026	0.025	0.026	0.029	0.030	0.199
TM7>TM7-3>CW040>F16>unclassified genus	0.025	0.009	0.024	0.018	0.016	0.018	0.015	0.022	0.010	0.012	0.007
Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>unclassified genus	0.023	0.016	0.023	0.021	0.023	0.020	0.020	0.024	0.025	0.027	0.126
Bacteroidetes>Bacteroidia>Bacteroidales>BS11>unclassified genus	0.022	0.017	0.026	0.021	0.020	0.018	0.013	0.027	0.017	0.018	0.097
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>YRC22	0.022	0.014	0.013	0.014	0.016	0.013	0.014	0.011	0.023	0.011	0.157
Verrucomicrobia>Verruco-5>WCHB1-41>RFP12>unclassified genus	0.020	0.021	0.023	0.025	0.017	0.021	0.016	0.017	0.028	0.026	0.004
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Ruminococcus	0.020	0.025	0.021	0.021	0.026	0.018	0.026	0.020	0.017	0.021	0.348
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>unclassified genus	0.016	0.016	0.023	0.016	0.025	0.015	0.015	0.022	0.018	0.023	0.057
Bacteroidetes>Bacteroidia>Bacteroidales>Prevotellaceae>Prevotella	0.014	0.027	0.020	0.023	0.026	0.017	0.023	0.028	0.022	0.016	0.135
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>BF311	0.014	0.005	0.009	0.008	0.006	0.008	0.010	0.005	0.004	0.006	0.005
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>CF231	0.012	0.018	0.009	0.011	0.010	0.010	0.013	0.006	0.011	0.007	0.022
Spirochaetes>Spirochaetes>Spirochaetales>Spirochaetaceae>Treponema	0.011	0.016	0.011	0.009	0.009	0.009	0.011	0.008	0.014	0.008	0.009
Verrucomicrobia>Verrucomicrobiae>Verrucomicrobiales>Verrucomicrobiaceae>Akkermansia	0.011	0.005	0.012	0.014	0.011	0.019	0.010	0.020	0.016	0.026	0.002
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Coprococcus	0.010	0.011	0.008	0.010	0.012	0.012	0.016	0.011	0.012	0.016	0.035
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	0.009	0.005	0.007	0.011	0.010	0.011	0.007	0.011	0.012	0.013	0.017
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Oscillospira	0.008	0.011	0.008	0.008	0.008	0.007	0.009	0.010	0.007	0.008	0.245
Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>p-75-a5	0.008	0.013	0.013	0.010	0.009	0.015	0.010	0.015	0.009	0.009	0.077
Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>unclassified genus	0.007	0.006	0.007	0.007	0.007	0.009	0.009	0.008	0.009	0.009	0.463
Firmicutes>Clostridia>Clostridiales>Christensenellaceae>unclassified genus	0.006	0.005	0.008	0.006	0.007	0.006	0.008	0.008	0.006	0.006	0.019
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>[Prevotella]	0.006	0.009	0.002	0.006	0.004	0.004	0.004	0.002	0.005	0.006	0.002
Bacteroidetes>Bacteroidia>Bacteroidales>RF16>unclassified genus	0.006	0.006	0.007	0.008	0.005	0.008	0.005	0.004	0.007	0.005	0.967

Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Clostridium	0.006	0.008	0.006	0.009	0.008	0.005	0.009	0.007	0.008	0.008	0.035
Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Phascolarctobacterium	0.005	0.011	0.005	0.007	0.011	0.005	0.009	0.005	0.009	0.010	0.031
Tenericutes>Mollicutes>RF39>unclassified family>unclassified genus	0.005	0.004	0.006	0.005	0.004	0.005	0.004	0.005	0.003	0.003	0.004
Firmicutes>Clostridia>Clostridiales>Veillonellaceae>unclassified genus	0.005	0.003	0.003	0.003	0.003	0.001	0.002	0.003	0.003	0.002	0.234
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.005	0.008	0.005	0.007	0.005	0.007	0.007	0.004	0.004	0.004	0.003
Cyanobacteria>4C0d-2>YS2>unclassified family>unclassified genus	0.005	0.004	0.004	0.003	0.004	0.003	0.003	0.004	0.002	0.002	0.007
Fibrobacteres>Fibrobacteria>Fibrobacterales>Fibrobacteraceae>Fibrobacter	0.004	0.003	0.004	0.003	0.005	0.003	0.003	0.001	0.004	0.002	0.098
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Pseudobutyrivibrio	0.004	0.004	0.003	0.005	0.004	0.004	0.004	0.004	0.005	0.004	0.843
Firmicutes>Clostridia>Clostridiales>Eubacteriaceae>Pseudoramibacter_Eubacterium	0.004	0.003	0.006	0.006	0.003	0.007	0.002	0.005	0.006	0.004	0.007
Spirochaetes>MVP-15>PL-11B10>unclassified family>unclassified genus	0.004	0.001	0.003	0.001	0.004	<0.001	0.003	0.002	0.002	0.001	0.028
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Blautia	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.004	0.005	0.008	0.365
Bacteroidetes>Bacteroidia>Bacteroidales>Porphyromonadaceae>Paludibacter	0.004	0.007	0.010	0.007	0.003	0.002	0.007	0.007	0.005	0.002	0.699
Firmicutes>Bacilli>Lactobacillales>Lactobacillaceae>Lactobacillus	0.003	0.003	0.005	0.005	0.003	0.004	0.007	0.008	0.005	0.017	0.276
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Sarcina	0.003	0.001	0.001	0.001	0.001	0.004	0.001	0.002	0.002	0.002	0.493
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.003	0.004	0.005	0.003	0.003	0.004	0.003	0.003	0.003	0.004	0.313
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>Bacteroides	0.003	0.004	0.005	0.007	0.004	0.003	0.003	0.003	0.006	0.003	0.308
Actinobacteria>Actinobacteria>Actinomycetales>Nocardiaceae>Rhodococcus	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.001	0.002	0.001	0.409
Euryarchaeota>Methanobacteria>Methanobacteriales>Methanobacteriaceae>Methanobrevibacter	0.002	0	0.001	0.001	0.001	0.001	0.001	0.001	<0.001	0.001	0.032
Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>RFN20	0.002	0.001	0.002	0.001	0.001	0.002	0.002	0.002	0.003	0.002	0.851
WPS-2>unclassified class>unclassified order>unclassified family>unclassified genus	0.002	0.001	0.002	0.001	0.001	0.004	0.002	0.002	0.002	0.001	0.049
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>unclassified genus	0.001	0.002	0.004	0.004	0.003	0.002	0.002	0.002	<0.001	0.001	0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Epulopiscium	0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.009
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>[Ruminococcus]	0.001	0.001	0.006	0.001	0.001	0.001	0.004	0.002	0.001	0.001	0.655
Proteobacteria>Gammaproteobacteria>Enterobacteriales>Enterobacteriaceae>unclassified genus	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.940
Fusobacteria>Fusobacteriia>Fusobacteriales>Fusobacteriaceae>Fusobacterium	<0.001	<0.001	<0.001	0.001	0	<0.001	<0.001	0	<0.001	<0.001	0.859
Verrucomicrobia>Verruco-5>LD1-PB3>unclassified family>unclassified genus	0	<0.001	0	0	0	0	<0.001	0.002	0	0	<0.001*
Other Genera <1%	0.042	0.042	0.044	0.042	0.043	0.055	0.052	0.042	0.047	0.04	0.046

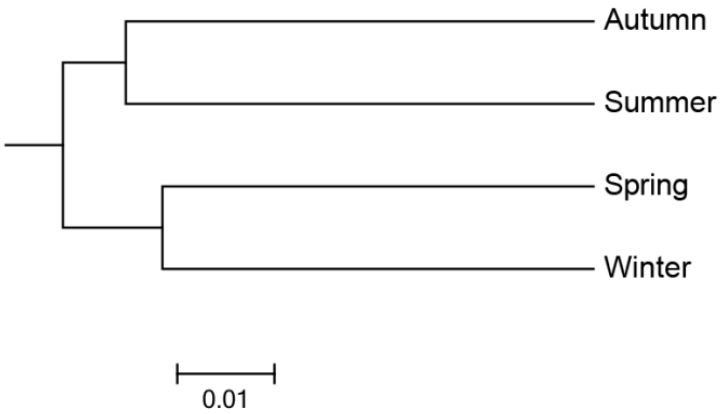
*Level of significance was P≤0.001 after Bonferroni correction for multiple comparisons

D-7. Figure S1. Principal coordinate analysis of the faecal bacterial community by season and month.



Legend: The 2-D plot in panel (A) shows the beta diversity of the faecal bacterial community of horses by season and panel (B) shows the beta diversity by month.

D-8. Figure S2. Season-wise hierarchial clustering of the faecal bacterial community of horses included in the study.



Legend: The cladogram shows the beta diversity of the bacterial community in the faecal samples and was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Seasons were categorised as follows: Summer – December, January, February; Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November.

D-9. An illustration of the seasonal variation in pasture during the study period.

(A) Horses grazing on good quality ‘lush’ pasture

(B) Pasture (limited pasture cover) supplemented with hay

(C) Dry pasture

D-10. Table S6. Comparison of mean relative abundance at the phylum level between seasons.

Taxon	Autumn	Winter	Spring	Summer	P value
Firmicutes	0.607	0.687	0.666	0.599	<0.001*
Bacteroidetes	0.234	0.183	0.215	0.244	0.008
Verrucomicrobia	0.042	0.037	0.028	0.040	<0.001*
Unassigned phyla	0.032	0.023	0.025	0.037	<0.001*
Actinobacteria	0.022	0.016	0.014	0.017	0.004
TM7	0.017	0.019	0.016	0.018	0.906
Spirochaetes	0.016	0.010	0.011	0.018	0.009
Proteobacteria	0.007	0.005	0.004	0.007	<0.001*
Tenericutes	0.006	0.005	0.005	0.005	0.752
Fibrobacteres	0.004	0.002	0.004	0.003	0.415
Cyanobacteria	0.004	0.003	0.003	0.004	0.01
Euryarchaeota	0.002	0.001	0.001	0.002	<0.001*
WPS-2	0.002	0.002	0.001	0.001	0.079
Fusobacteria	<0.001	<0.001	<0.001	<0.001	0.107
Other Phyla <1%	0.007	0.007	0.007	0.007	0.636

Legend: *Level of significance was P≤0.003 after Bonferroni correction for multiple comparisons. Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November; Summer – December, January, February .

D-11. Table S7. Comparison of mean relative abundance at the genus level between seasons.

Taxon	Autumn	Winter	Spring	Summer	P value
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>unclassified genus	0.187	0.226	0.233	0.223	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.144	0.139	0.138	0.121	0.088
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.117	0.144	0.134	0.109	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>unclassified family>unclassified genus	0.105	0.075	0.101	0.114	<0.001*
Unassigned genera	0.032	0.023	0.025	0.037	<0.001*
Verrucomicrobia>Verruco-5>WCHB1-41>RFP12>unclassified genus	0.024	0.016	0.022	0.024	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>Prevotellaceae>Prevotella	0.024	0.02	0.024	0.018	0.272
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Ruminococcus	0.022	0.024	0.023	0.017	0.018
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>unclassified genus	0.021	0.024	0.015	0.017	0.146
Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>unclassified genus	0.019	0.021	0.027	0.021	<0.001*
Verrucomicrobia>Verrucomicrobiae>Verrucomicrobiales>Verrucomicrobiaceae>Akkermansia	0.018	0.02	0.006	0.014	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>CF231	0.017	0.005	0.011	0.01	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>YRC22	0.017	0.017	0.015	0.012	0.384
Bacteroidetes>Bacteroidia>Bacteroidales>BS11>unclassified genus	0.016	0.02	0.021	0.022	0.16
TM7>TM7-3>CW040>F16>unclassified genus	0.016	0.019	0.016	0.017	0.886
Spirochaetes>Spirochaetes>Spirochaetales>Spirochaetaceae>Treponema	0.014	0.008	0.01	0.011	0.06
Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>p-75-a5	0.011	0.01	0.013	0.01	0.035
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Coprococcus	0.011	0.014	0.011	0.011	0.051
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	0.009	0.014	0.009	0.006	<0.001*
Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Phascolarctobacterium	0.009	0.007	0.005	0.01	0.023
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Clostridium	0.008	0.007	0.01	0.006	<0.001*
Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>unclassified genus	0.008	0.008	0.008	0.006	0.01
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Oscillospira	0.008	0.01	0.007	0.008	0.177
Firmicutes>Bacilli>Lactobacillales>Lactobacillaceae>Lactobacillus	0.007	0.012	0.002	0.002	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>BF311	0.007	0.006	0.008	0.009	0.006
Bacteroidetes>Bacteroidia>Bacteroidales>RF16>unclassified genus	0.006	0.001	0.004	0.012	<0.001*

Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.006	0.004	0.007	0.005	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>[Prevotella]	0.006	0.004	0.004	0.005	0.097
Bacteroidetes>Bacteroidia>Bacteroidales>Porphyromonadaceae>Paludibacter	0.005	0.001	0.003	0.013	<0.001*
Firmicutes>Clostridia>Clostridiales>Christensenellaceae>unclassified genus	0.005	0.008	0.007	0.006	0.005
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Pseudobutyrivibrio	0.005	0.003	0.004	0.004	0.279
Tenericutes>Mollicutes>RF39>unclassified family>unclassified genus	0.005	0.005	0.004	0.004	0.343
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.004	0.004	0.003	0.004	0.002
Cyanobacteria>4C0d-2>YS2>unclassified family>unclassified genus	0.004	0.003	0.003	0.004	0.007
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>Bacteroides	0.004	0.004	0.004	0.004	0.014
Firmicutes>Clostridia>Clostridiales>Eubacteriaceae>Pseudoramibacter_Eubacterium	0.004	0.004	0.006	0.005	0.122
Fibrobacteres>Fibrobacteria>Fibrobacterales>Fibrobacteraceae>Fibrobacter	0.004	0.002	0.004	0.003	0.415
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Blautia	0.004	0.004	0.004	0.005	0.831
Firmicutes>Clostridia>Clostridiales>Veillonellaceae>unclassified genus	0.003	0.005	0.002	0.001	<0.001*
Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Sarcina	0.002	0.004	0.001	0	<0.001*
Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>RFN20	0.002	0.001	0.002	0.003	<0.001*
Actinobacteria>Actinobacteria>Actinomycetales>Nocardiaceae>Rhodococcus	0.002	0.001	0.001	0.003	<0.001*
Euryarchaeota>Methanobacteria>Methanobacteriales>Methanobacteriaceae>Methanobrevibacter	0.002	0.001	0.001	0.001	0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>unclassified genus	0.002	0.002	0.003	0.002	0.061
WPS-2>unclassified class>unclassified order>unclassified family>unclassified genus	0.002	0.002	0.001	0.001	0.079
Spirochaetes>MVP-15>PL-11B10>unclassified family>unclassified genus	0.001	0.001	0.001	0.005	0.002
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>[Ruminococcus]	0.001	0.005	0.001	0.001	0.917
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Epulopiscium	<0.001	0.001	<0.001	<0.001	<0.001*
Proteobacteria>Gammaproteobacteria>Enterobacteriales>Enterobacteriaceae>unclassified genus	<0.001	<0.001	<0.001	<0.001	0.019
Fusobacteria>Fusobacteriia>Fusobacteriales>Fusobacteriaceae>Fusobacterium	<0.001	<0.001	<0.001	<0.001	0.056
Verrucomicrobia>Verruco-5>LD1-PB3>unclassified family>unclassified genus	<0.001	<0.001	<0.001	0.001	0.511
Other Genera <1%	0.052	0.039	0.039	0.051	<0.001*

Legend: *Level of significance was P≤0.001 after Bonferroni correction for multiple comparisons. Autumn – March, April, May; Winter – June, July, August; Spring – September, October, November; Summer – December, January, February.

D-12. Table S8. Comparison of mean relative abundance at the phylum level between months.

Taxon	Apr (2013)	May (2013)	Jun (2013)	Jul (2013)	Aug (2013)	Sep (2013)	Oct (2013)	Nov (2013)	Dec (2013)	Jan (2014)	Feb (2014)	Mar (2014)	P value
Firmicutes	0.656	0.664	0.677	0.697	0.688	0.668	0.673	0.656	0.662	0.600	0.534	0.508	<0.001*
Bacteroidetes	0.198	0.169	0.179	0.182	0.189	0.220	0.200	0.223	0.185	0.249	0.298	0.328	<0.001*
Unassigned phyla	0.024	0.029	0.023	0.022	0.022	0.022	0.026	0.026	0.025	0.044	0.041	0.042	<0.001*
Verrucomicrobia	0.039	0.050	0.050	0.027	0.032	0.025	0.032	0.028	0.040	0.038	0.041	0.039	0.003*
Spirochaetes	0.012	0.007	0.010	0.009	0.010	0.014	0.009	0.012	0.008	0.018	0.027	0.027	<0.001*
TM7	0.017	0.028	0.018	0.017	0.021	0.014	0.017	0.018	0.032	0.012	0.009	0.005	<0.001*
Actinobacteria	0.029	0.022	0.015	0.020	0.014	0.011	0.016	0.014	0.023	0.010	0.017	0.014	<0.001*
Proteobacteria	0.006	0.006	0.006	0.005	0.003	0.004	0.004	0.003	0.005	0.008	0.009	0.008	<0.001*
Tenericutes	0.004	0.009	0.006	0.004	0.006	0.005	0.005	0.006	0.006	0.005	0.005	0.006	0.009
Cyanobacteria	0.003	0.003	0.002	0.002	0.005	0.003	0.003	0.003	0.004	0.003	0.005	0.005	0.001*
Fibrobacteres	0.002	<0.001	0.002	0.002	0.003	0.006	0.002	0.003	0.001	0.002	0.005	0.009	<0.001*
Euryarchaeota	0.004	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.001*
WPS-2	<0.001	0.004	0.003	0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.001	<0.001	0.008
Fusobacteria	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.082
Other Phyla <1%	0.007	0.007	0.006	0.008	0.006	0.006	0.008	0.007	0.007	0.008	0.006	0.007	0.206

*Level of significance was P≤0.003 after Bonferroni correction for multiple comparisons

D-13. Table S9. Comparison of mean relative abundance at the genus level between months.

Taxon	Apr (2013)	May (2013)	Jun (2013)	Jul (2013)	Aug (2013)	Sep (2013)	Oct (2013)	Nov (2013)	Dec (2013)	Jan (2014)	Feb (2014)	Mar (2014)	P value
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae >unclassified genus	0.166	0.230	0.219	0.232	0.228	0.251	0.237	0.212	0.234	0.248	0.186	0.169	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>unclassified family>unclassified genus	0.089	0.066	0.063	0.077	0.086	0.108	0.098	0.099	0.093	0.116	0.135	0.157	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >unclassified genus	0.182	0.137	0.138	0.139	0.140	0.129	0.134	0.153	0.133	0.114	0.118	0.112	0.006
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.135	0.127	0.138	0.149	0.144	0.131	0.136	0.134	0.134	0.100	0.094	0.089	<0.001*
Unassigned genera	0.024	0.029	0.023	0.022	0.022	0.022	0.026	0.026	0.025	0.044	0.041	0.042	<0.001*
Verrucomicrobia>Verruco-5>WCHB1-41>RFP12 >unclassified genus	0.024	0.018	0.015	0.014	0.019	0.019	0.026	0.02	0.023	0.028	0.022	0.028	<0.001*
Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae] >unclassified genus	0.023	0.020	0.018	0.020	0.026	0.024	0.032	0.024	0.029	0.020	0.015	0.014	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>RF16 >unclassified genus	0.001	0.001	0.001	<0.001	0.003	0.006	0.004	0.003	0.002	0.019	0.016	0.016	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales >Porphyromonadaceae>Paludibacter	<0.001	<0.001	0.001	0.001	0.003	0.005	0.002	0.001	0.002	0.019	0.016	0.013	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales>BS11 >unclassified genus	0.009	0.025	0.024	0.022	0.015	0.019	0.026	0.019	0.022	0.019	0.024	0.017	0.014
Bacteroidetes>Bacteroidia>Bacteroidales >Prevotellaceae>Prevotella	0.023	0.009	0.019	0.021	0.021	0.020	0.018	0.034	0.013	0.015	0.027	0.038	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales >[Paraprevotellaceae]>unclassified genus	0.014	0.028	0.026	0.035	0.011	0.016	0.013	0.015	0.016	0.015	0.020	0.021	0.012
Firmicutes>Clostridia>Clostridiales>Ruminococcaceae >Ruminococcus	0.018	0.026	0.028	0.025	0.020	0.023	0.017	0.027	0.014	0.014	0.024	0.024	0.001*
Firmicutes>Clostridia>Clostridiales>Veillonellaceae >Phascolarctobacterium	0.010	0.003	0.005	0.007	0.008	0.007	0.003	0.006	0.005	0.013	0.012	0.015	<0.001*
TM7>TM7-3>CW040>F16>unclassified genus	0.017	0.028	0.018	0.017	0.021	0.014	0.017	0.017	0.031	0.012	0.009	0.005	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales >[Paraprevotellaceae]>YRC22	0.017	0.019	0.019	0.015	0.016	0.015	0.012	0.017	0.008	0.012	0.016	0.015	0.06
Bacteroidetes>Bacteroidia>Bacteroidales >Bacteroidaceae>BF311	0.003	0.007	0.003	0.003	0.011	0.010	0.007	0.008	0.008	0.011	0.009	0.010	<0.001*

Firmicutes>Clostridia>Clostridiales>Ruminococcaceae >Oscillospira	0.008	0.008	0.008	0.016	0.007	0.009	0.007	0.007	0.007	0.011	0.008	0.007	0.002
Bacteroidetes>Bacteroidia>Bacteroidales >[Paraprevotellaceae]>CF231	0.029	0.004	0.005	0.002	0.009	0.009	0.010	0.014	0.010	0.009	0.011	0.015	<0.001*
Spirochaetes>Spirochaetes>Spirochaetales >Spirochaetaceae>Treponema	0.012	0.006	0.009	0.007	0.008	0.011	0.007	0.010	0.006	0.009	0.017	0.023	<0.001*
Verrucomicrobia>Verrucomicrobiae>Verrucomicrobial es>Verrucomicrobiaceae>Akkermansia	0.014	0.031	0.035	0.012	0.012	0.005	0.005	0.008	0.014	0.009	0.018	0.010	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >Coprococcus	0.013	0.011	0.014	0.013	0.016	0.013	0.009	0.012	0.013	0.009	0.012	0.008	0.004
Firmicutes>Erysipelotrichi>Erysipelotrichales >Erysipelotrichaceae>p-75-a5	0.013	0.013	0.008	0.010	0.012	0.010	0.019	0.011	0.018	0.008	0.005	0.007	<0.001*
Spirochaetes>MVP-15>PL-11B10>unclassified family>unclassified genus	<0.001	0	0.001	0.002	0.002	0.001	0.001	0.001	0	0.006	0.009	0.003	<0.001*
Firmicutes>Clostridia>Clostridiales>Clostridiaceae >unclassified genus	0.009	0.013	0.020	0.009	0.013	0.010	0.012	0.006	0.008	0.006	0.005	0.005	<0.001*
Firmicutes>Clostridia>Clostridiales>Christensenellacea e>unclassified genus	0.005	0.006	0.005	0.011	0.007	0.006	0.008	0.006	0.008	0.006	0.005	0.005	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >unclassified genus	0.008	0.004	0.004	0.003	0.006	0.006	0.007	0.008	0.005	0.005	0.005	0.006	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >Blautia	0.007	0.003	0.005	0.003	0.006	0.005	0.003	0.005	0.006	0.005	0.005	0.003	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales >[Paraprevotellaceae]>[Prevotella]	0.005	0.003	0.006	0.003	0.004	0.005	0.002	0.004	0.002	0.005	0.009	0.009	<0.001*
Firmicutes>Erysipelotrichi>Erysipelotrichales >Erysipelotrichaceae>RFN20	0.001	<0.001	0.001	<0.001	0.002	0.002	0.002	0.001	0.002	0.004	0.003	0.006	<0.001*
Actinobacteria>Coriobacteriia>Coriobacteriales >Coriobacteriaceae>unclassified genus	0.011	0.010	0.006	0.010	0.009	0.007	0.010	0.007	0.010	0.004	0.004	0.004	<0.001*
Firmicutes>Clostridia>Clostridiales>Clostridiaceae >Clostridium	0.010	0.007	0.005	0.007	0.008	0.009	0.009	0.011	0.007	0.004	0.005	0.008	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales >Bacteroidaceae>Bacteroides	0.002	0.002	0.009	0.002	0.002	0.004	0.004	0.003	0.003	0.004	0.006	0.007	<0.001*
Firmicutes>Clostridia>Clostridiales>Eubacteriaceae >Pseudoramibacter_Eubacterium	0.005	0.003	0.002	0.003	0.006	0.004	0.010	0.004	0.009	0.004	0.002	0.003	<0.001*
Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.005	0.003	0.003	0.004	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.003	0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >Pseudobutyrivibrio	0.007	0.003	0.004	0.002	0.004	0.004	0.004	0.005	0.003	0.004	0.004	0.004	0.046

Firmicutes>Bacilli>Lactobacillales>Lactobacillaceae >Lactobacillus	0.003	0.019	0.018	0.008	0.009	0.004	0.001	0.002	0.004	0.003	0.001	0	<0.001*
Tenericutes>Mollicutes>RF39>unclassified family>unclassified genus	0.003	0.009	0.006	0.004	0.005	0.004	0.004	0.005	0.005	0.003	0.003	0.002	<0.001*
Cyanobacteria>4C0d-2>YS2>unclassified family>unclassified genus	0.003	0.002	0.002	0.002	0.004	0.003	0.003	0.003	0.003	0.003	0.005	0.005	<0.001*
Fibrobacteres>Fibrobacteria>Fibrobacterales >Fibrobacteraceae>Fibrobacter	0.002	0	0.002	0.002	0.003	0.006	0.002	0.003	0.001	0.002	0.005	0.009	<0.001*
Bacteroidetes>Bacteroidia>Bacteroidales >Bacteroidaceae>unclassified genus	0.002	0.001	0.001	<0.001	0.005	0.002	0.003	0.003	0.002	0.002	0.003	0.003	0.002
WPS-2>unclassified class>unclassified order>unclassified family>unclassified genus	<0.001	0.004	0.003	0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.001	<0.001	0.008
Firmicutes>Clostridia>Clostridiales>Clostridiaceae >Sarcina	<0.001	0.006	0.005	0.003	0.003	0.001	0.001	0.001	0.001	0.001	<0.001	<0.001	<0.001*
Actinobacteria>Actinobacteria>Actinomycetales >Nocardiaceae>Rhodococcus	0.002	0.002	0.002	0.001	0.001	<0.001	0.001	0.001	0.003	0.001	0.004	0.003	<0.001*
Firmicutes>Clostridia>Clostridiales>Veillonellaceae >unclassified genus	0.003	0.004	0.005	0.006	0.003	0.002	0.002	0.002	0.001	0.001	0.002	0.002	<0.001*
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >[Ruminococcus]	0.001	<0.001	0.007	0.007	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.026
Firmicutes>Clostridia>Clostridiales>Lachnospiraceae >Epulopiscium	<0.001	<0.001	0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001*
Euryarchaeota>Methanobacteria>Methanobacteriales >Methanobacteriaceae>Methanobrevibacter	0.003	0.001	0.001	0.001	<0.001	0.001	0.001	<0.001	0.001	<0.001	<0.001	0.001	<0.001*
Proteobacteria>Gammaproteobacteria>Enterobacteriales >Enterobacteriaceae>unclassified genus	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0	<0.001	<0.001	<0.001	<0.001	0.002
Fusobacteria>Fusobacteriia>Fusobacteriales >Fusobacteriaceae>Fusobacterium	0	<0.001	0.001	<0.001	0	0	0	0	<0.001	<0.001	<0.001	0	0.039
Verrucomicrobia>Verruco-5>LD1-PB3>unclassified family>unclassified genus	0	0	0	<0.001	<0.001	0	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.879
Other Genera <1%	0.058	0.047	0.038	0.043	0.033	0.035	0.043	0.038	0.048	0.048	0.056	0.051	<0.001*

*Level of significance was P≤0.001 after Bonferroni correction for multiple comparisons

CHAPTER 7

FEEDING BEHAVIOUR AND DIGESTA TRANSIT TIME IN STABLED HORSES

(Feed and Marker Trial)

PRELUDE TO CHAPTER 7

Horses and ponies in New Zealand are kept on pasture all year round (Chapter 3) and many appear to maintain their body weight and a higher than normal body condition (Chapter 4), even though the quality and availability of pasture changes over seasons (Chapter 6). The capability to satisfy energy requirements across variable planes of nutrition could be associated with the level of feed intake and transit time of digesta, which potentially affects the time available for microbial fermentation in the hindgut. In Chapter 5, a significant change in bacterial diversity was detected in the faeces of horses within four days following abrupt dietary change. However, since the faecal samples were only collected at 4-day intervals, the change in diversity must have been initiated earlier than four days, which was perhaps associated with the transit time of the diet consumed. Similarly, an acute shift in the proportion of bacterial phyla observed in Chapter 6 may have been associated with differences in the feeding behaviour between individual horses (some horses eat less than others), the quantity of feed consumed (dry versus lush pasture) and the passage rate of digesta through the gastrointestinal tract.

Chapter 7 is the first part of the feeding trial, where differences in gastrointestinal transit times of four different forage-based diets were investigated. The horses included in this trial were administered with indigestible polyethylene markers via a nasogastric tube at the start of each dietary treatment block. The feeding behaviour, rate of faecal voiding and marker retrieval from the faeces was recorded to determine differences in the gastrointestinal transit times across diets and horses.

Supplementary information on the experimental design and some additional results are presented in the appendix for this chapter (Appendix E). Chapter 7 is based on a manuscript submitted to BMC Veterinary Research (BMC Vet Res).

Fernandes, K.A., Rogers, C.W., Gee, E.K., Bolwell, C.F., Fitch, G., Kittelmann, S., Bermingham, E.N. and Thomas, D.G. (Submitted 30.01.2016). Comparison of gastrointestinal transit times in stabled Thoroughbred horses during abrupt dietary transition between freshly cut pasture and three conserved forage-based diets. *BMC Vet Res*.

CHAPTER 7: COMPARISON OF FEEDING BEHAVIOUR AND GASTROINTESTINAL TRANSIT TIMES IN STABLED THOROUGHBRED HORSES FOLLOWING ABRUPT DIETARY TRANSITION BETWEEN FRESHLY CUT PASTURE AND THREE CONSERVED FORAGE-BASED DIETS

7.1 Abstract

The type of forage offered to horses varies in physical form, moisture content and nutrient quality, and these variables could affect the intake, passage rate and digestibility of the forage consumed. The current study aimed to investigate the changes in passage rate of digesta through the gastrointestinal tract in horses fed four different forage-based diets (diet effect), and to determine the effects of individual variation between the horses (horse effect). Thoroughbred mares (n=6) were stabled in loose-boxes for six weeks. During weeks 1, 3 and 5 (washout periods), all horses were fed freshly cut pasture, either in restricted quantities (week 1) or *ad libitum* (weeks 3 and 5). Using a 3 × 3 Latin square design during weeks 2, 4 and 6, each pair of horses was abruptly transitioned to one of three conserved forage-based diets (chopped ensiled forage containing HNF® High Nutritional Fiber fed exclusively or with oats, or perennial ryegrass hay with oats) fed *ad libitum*. At the beginning of each week, indigestible polyethylene markers were administered to the horses *via* a nasogastric tube, followed immediately by transition to the new diet. Data on the feeding behaviour, feed intake and frequency of voiding faeces were recorded, and total faecal matter was collected at hourly intervals to manually retrieve the polyethylene markers. All horses remained in good health and maintained body weight and condition throughout the study period. There was a significant diet effect on the daily dry matter intake of feed ($P<0.0001$), percentage of time spent eating ($P<0.001$), frequency of voiding faeces ($P<0.05$) and quantity of faeces voided ($P<0.0001$). There was a significant horse effect on the daily dry matter intake of feed ($P<0.0001$) and quantity of faeces voided ($P<0.0001$), but no differences in the percentage of time spent eating or the frequency of voiding faeces. There were significant diet and horse effects on the cumulative percentage of markers recovered ($P<0.001$ and $P<0.001$, respectively), the time to recovery of the first marker in the faeces ($P<0.01$ and $P<0.01$,

respectively) and the mean retention time of markers in the gastrointestinal tract ($P<0.05$ and $P<0.001$, respectively). Mean retention time was negatively correlated with feed intake and quantity of faeces voided (R^2=-0.51 and R^2=-0.64, respectively). Diet- and horse-specific factors influenced the passage rate of digesta through the gastrointestinal tract. The level of feed intake appeared to drive the passage rate of digesta, and indirectly the apparent digestibility of the feed consumed.

7.2 Introduction

Horses are hindgut fermenters adapted to eating a high forage diet (Janis, 1976). When kept on pasture, horses spend most (70%) of their time grazing a variety of forage species (Crowell-Davis *et al.*, 1985, Randall *et al.*, 2014). In New Zealand, most horses are kept on pasture all year round (Fernandes *et al.*, Verhaar *et al.*, 2014), but it is common practice to intensively manage racehorses and some competition horses in stables for parts of the day, with variable turnout periods for grazing (Stowers *et al.*, Rogers *et al.*, 2007). A variety of conserved forages, grain and grain-by-products, are frequently incorporated into the diet of many horses, especially when pasture is unavailable or inadequate in quantity and nutritive value, or to provide additional dietary energy for performance (Goodwin *et al.*, 2002, Harris, 2009, Hoffman *et al.*, 2009). Gastrointestinal disorders may occur in horses and ponies, with some animals more prone than others (Clarke *et al.*, 1990, Hudson *et al.*, 2001, Julliand, 2005). A common recommendation to prevent gastrointestinal disturbances in horses is to avoid abrupt dietary changes and to maintain high proportions of forage in the diet (Julliand *et al.*, 2001). Although several studies have investigated aspects of conserved forages fed to horses (Drogoul *et al.*, 2001, Müller *et al.*, 2008, Muhonen *et al.*, 2009, Muhonen *et al.*, 2010), the effect of different conserved forages on the passage rate of digesta and the microbial population in the hindgut is poorly understood.

Many types of conserved forages are fed to horses which vary in physical form, e.g. long-stemmed, chaffed or pelleted forages, the nutrient quality of which can vary depending on several factors (Müller and Udén, 2007). For example, conserved forages may consist of variable grass and legume species such as ryegrass, clover, timothy, lucerne or a blend of multiple forage species, and these may be harvested at different stages of maturity (i.e. early, middle or late stages), resulting in variable nutrient quality of the forage. Furthermore, the forage may be preserved by different methods (sun dried, ensiled and chopped or pelleted),

resulting in variable moisture levels (e.g. dry hay vs. moist haylage) and energy content (e.g. high energy in haylage vs. low energy in mature hay) (Lewis *et al.*, 1995, Hoffman *et al.*, 2001, Müller and Udén, 2007).

Traditionally, the most common method for conservation of forage was in the form of hay. More recently, feeding horses ensiled/fermented forages (such as haylage) is becoming increasingly popular (Müller and Udén, 2007). Several authors have reported potential benefits associated with ensiled/fermented forages including higher voluntary feed intake, palatability and digestibility (Müller and Udén, 2007, Ragnarsson and Lindberg, 2010), reduced dust particles due to the higher moisture content (Clements and Pirie, 2007), and easier transportation and convenient storage due to the smaller size of polyethylene-wrapped packaging (Patel *et al.*, 2014, Verhaar *et al.*, 2014) when compared to hay.

Passage rate of digesta (feed) through the gastrointestinal tract has been previously investigated, and is best described by measuring the mean retention time (MRT) of indigestible markers that travel through the gastrointestinal tract with the feed consumed (Pearson and Merritt, 1991, Cuddeford *et al.*, 1995, Pearson *et al.*, 2001). The transit time (T_1) is described as the time when the first marker is recovered in the faeces, and is an indication of the minimum time between ingestion of the feed and voiding of faeces (Udén *et al.*, 1982).

Significant differences in the passage rate of digesta through the gastrointestinal tract have been reported in equids (e.g. horses, ponies and donkeys) fed different diets, i.e. 24-26 h when grazing or fed cut pasture (Grace *et al.*, 2003) vs. 26-77 h when fed different types of conserved forages (Cuddeford *et al.*, 1995, Pearson *et al.*, 2001, Moore-Colyer *et al.*, 2003), and 43-52 h when fed ground pelleted hay vs. 27-46 h when fed chopped hay (Drogoul *et al.*, 2000a). The physical form of the forage and the particle size of digesta were identified as dominant effects (Drogoul *et al.*, 2000a, Moore-Colyer *et al.*, 2003). Furthermore, increasing the proportion of forage in the diet at the expense of grain reduced the passage rate of digesta through the gastrointestinal tract, from 42 h for a 50:50 hay and barley diet, to 30 h for a 100% hay diet (Drogoul *et al.*, 2001). In ponies fed *ad libitum* vs. restricted quantities, the passage rate increased from 21 to 31 h for chopped alfalfa, and from 32 to 36 h for chopped oat straw (Pearson *et al.*, 2001). However, while the passage rate of different types of forage and mixed forage-grain diets has been investigated in horses, following an adaption period after dietary change, the passage rate (MRT and T_1) immediately following abrupt transition between forage-based diets has not been investigated.

The current study aimed to investigate the changes in T_1 and MRT when horses were fed four different forage-based diets (diet effect), and to determine the effects of individual variation between horses (horse effect) on the passage rate by using indigestible markers. It was hypothesised that the passage rate in horses fed pasture would be shorter than in horses fed the conserved forage diets, and there would be significant differences in the feed intake and passage rate between Thoroughbred horses managed similarly on the same diet.

7.3 Materials and Methods

7.3.1 Ethics statement

The use of animals, experimental procedures and collection of the faecal samples for the study, were approved by the Massey University Animal Ethics Committee (MUAEC), Massey University, Palmerston North, New Zealand (Protocol number 14/35). The management of the horses used in the study (including feeding, housing, husbandry and welfare) were in accordance with the guidelines set within the code of ethical conduct for the use of live animals for research, testing and teaching. A veterinarian examined the horses on a weekly basis to ensure that all horses remained clinically normal during the study period.

7.3.2 Experimental design

The study was conducted from 14th July to 24th August 2014 (42 d in winter), with the experimental period sub-divided into six dietary treatment blocks, each of seven days duration. Using a 3 × 3 replicated Latin square design, stabled horses were abruptly transitioned to three randomly-allocated conserved forage-based diets. Between dietary treatment periods, the horses were provided with a washout period of seven days, during which freshly cut pasture was fed *ad libitum* to each horse (Appendix E-1). Feed and faecal samples were collected at regular intervals following each dietary transition, and data were recorded to investigate the differences in gastrointestinal transit times between horses and diets.

7.3.3 Selection of horses and pre-trial management

Six Thoroughbred mares were enrolled in the study (mean age ± standard deviation (SD), 13.5 ± 3.7 years and mean body weight (BW) ± SD, 528 ± 26 kg, weighed at the beginning of the study). Prior to the study, the horses were maintained on a commercial Thoroughbred stud farm (Palmerston North, Manawatu, New Zealand), and managed as a

cohort on predominantly perennial ryegrass-clover pasture (typically ~80-95% perennial ryegrass (*Lolium perenne*) and ~5-20% white clover (*Trifolium repens*) (Hoskin and Gee, 2004)). The horses had received an annual vaccination (Equivac 2 in 1, Pfizer Animal Health, Australia) and the most recent anthelmintic treatment had been administered one week prior to commencement of the study. At the start of the study, all horses were of similar body condition (body condition score [BCS] five on a 9-point scale (Henneke *et al.*, 1983)), as assessed by the author (KAF). The horses were reported to have been in good health during the six months preceding the study, and had not received antibiotic treatments during this period.

Three days before the start of the study, the horses were transported from the stud farm to the trial site (Veterinary Teaching Hospital, Massey University, Palmerston North, New Zealand; distance ~20 km; travel time ~30 min). On arrival, the horses were transferred into individual, adjacent, outdoor paddocks (15 × 15 m, containing ryegrass-clover pasture) to facilitate adaptation to the new environment. The general behaviour of the horses was observed for signs of stress, pain, inappetence and discomfort. A fresh faecal sample was collected (from a recently voided faecal mass) from each horse within 2 h of arrival, a sub-sample was examined for faecal egg count and the remaining portion was snap frozen in liquid nitrogen and stored at -80°C (Day 0 faecal sample). During the three-day adaptation period, each horse was offered ~12 kg DM (dry matter) of ryegrass-clover hay per day, fed twice daily, in addition to the limited amount of grazing available in the paddocks.

7.3.4 Housing and stable management during the trial

On Day 1 of the trial, the horses were individually stabled in 3 × 3.5 m loose-boxes, with sawdust bedding to a depth of 8-10 cm. The loose-boxes were adjacent to each other and the top half of the stable door was kept open at all times, allowing visual contact between all horses. The horses were turned-out daily during the trial into two 6 × 8 m concrete yards, individually, for 30 min in the morning and afternoon. The yards were adjacent to each other allowing each pair of horses visual, tactile and olfactory communication, also allowing them to engage in mutual grooming over the fence. Horses that showed stereotypic behaviours (weaving or box-walking) in the stables were hand-walked for a short period or turned out in the yard for an extended period of time (when required), without altering the feeding regimen. During each morning turn-out period, the loose-boxes were cleaned, with wet bedding, faecal

matter and refused feed removed, and bedding, water and feed were replenished. The horses were groomed regularly and were covered with rugs during the trial (Appendix E-2).

The horses were provided *ad libitum* access to water in a 40 l bucket (Italio flexi tub, Auckland, New Zealand) and a 500 g trace-mineral salt block (Summit Littlix multi-mineral salt block, Dominion Salt Ltd., Mount Maunganui, New Zealand). Cut grass and hay was provided in hay nets (large size nylon rope hay net, 107 cm length with 15 x 15 cm mesh openings; Shires Equestrian Products, Herefordshire, United Kingdom) and the chopped ensiled forage diet, oats and some cut pasture were provided in a feed bucket (40 L capacity, Italio flexi tub). The hay nets were suspended from a carabiner attached to the upper corner of the stable door and the feed buckets were placed in the corner adjacent to the door. This arrangement allowed for easy access to the feed by both the horse and the personnel, thereby enabling provision of feed and collection of refusals or spillage with minimal disruption to the horse when it was boxed (Appendix E-3).

7.3.5 Selection of diets and feeding management

The horses were offered four types of forage-based diets over the 6-week period. Freshly cut pasture (Diet P) was fed to the horses as a washout diet between the dietary treatment blocks (Diets P1, P2 and P3 fed during weeks 1, 3 and 5 of the trial, respectively). The three treatment diets were: a commercial chopped ensiled forage containing HNF® High Nutritional Fiber (Diet FE), the same commercial chopped ensiled forage mixed with whole oats (Diet FE+O), and a perennial ryegrass hay fed with whole oats (Diet H+O). The nutrient composition of the dietary components is provided in Table 7.1.

Table 7.1. Nutrient analysis (on a DM basis) of dietary components used in the study on passage rate of digesta in horses.

	P1	P2	P3	FE	H	O
DE MJ/kg†	10.8 ± 0.4	11.0 ± 0.4	10.7 ± 0.3	10.7 ± 0.3	9.3 ± 0.3	15.1 ± 0.2
% DM	15.6 ± 1.7	16.4 ± 2.5	15.8 ± 2.4	39.5 ± 1.4	80.5 ± 1.3	83.4 ± 1.1
% Ash	13.2 ± 1.0	11.8 ± 0.8	12.2 ± 1.3	7.8 ± 0.5	7.6 ± 0.6	3.0 ± 0.0
% Crude Protein	25.0 ± 1.6	22.9 ± 1.7	19.2 ± 2.7	17.3 ± 1.3	11.7 ± 0.7	10.9 ± 0.2
% Crude Fat	4.9 ± 0.2	4.7 ± 0.4	4.4 ± 0.6	3.9 ± 0.3	1.9 ± 0.4	5.9 ± 0.2
% CHO‡	56.9 ± 2.2	60.7 ± 2.2	64.3 ± 4.1	71.0 ± 1.6	78.7 ± 1.3	80.3 ± 0.3
% ADF	45.7 ± 2.3	46.6 ± 2.4	49.3 ± 1.8	58.1 ± 3.1	62.9 ± 2.2	24.4 ± 2.1
% NDF	26.6 ± 1.6	28.2 ± 1.3	29.8 ± 1.9	39.1 ± 1.3	43.7 ± 2.2	12.1 ± 1.0
% Lignin	3.7 ± 0.7	4.8 ± 1.1	4.9 ± 1.0	6.4 ± 0.3	7.5 ± 0.6	2.5 ± 0.3
% Starch	1.0 ± 1.0	1.4 ± 0.9	0.5 ± 0.5	2.6 ± 0.6	1.3 ± 0.4	50.3 ± 3.2
% WSC	20.6 ± 1.5	21.8 ± 2.9	23.4 ± 2.4	10 ± 1.6	9.0 ± 1.2	NA
% ESC	13.9 ± 2.9	13.9 ± 3.9	17.7 ± 1.7	5.4 ± 1.2	6.6 ± 1.3	NA
% NFC§	30.3 ± 3.0	32.5 ± 2.2	34.5 ± 4.7	31.9 ± 1.6	35.0 ± 1.3	68.2 ± 1.3
% TDNϕ	70.8 ± 2.2	70.1 ± 2.4	68.3 ± 2.2	68.1 ± 1.5	59.1 ± 1.0	86.6 ± 1.3
RFV	169 ± 11	163 ± 10	151 ± 7	119 ± 8	94 ± 3	NA

Legend: The dietary components used in the study were P1, P2 and P3 - cut pasture fed in weeks 1, 2 and 3, respectively; FE - ensiled timothy and lucerne forage; H - perennial ryegrass hay with small proportions of clover, herbs and weeds; O - whole oats. DM - Dry matter; ADF - acid detergent fibre; NA – not applicable; NDF - neutral detergent fibre; WSC - water soluble carbohydrates; ESC - ethanol soluble carbohydrates; RFV- relative feed value.

†Digestible energy (DE) = DE (kcal/kg) = 255 + 3660 x TDN (converted to MJ/kg by multiplying by 0.004184)
‡Total carbohydrates (CHO) = 100 - (CP + fat + ash)
§ Non-fibre carbohydrates (NFC) or non-structural carbohydrates (NSC) = 100 - (CP + fat + ash + NDF)
ϕTotal digestible nutrients (TDN) = CP + (fat x 2.25) + NDF + NSC

During weeks 1, 3 and 5 of the trial, the horses were fed freshly cut grass obtained from ryegrass-clover pasture (typically comprising of ~80-95% perennial ryegrass (*Lolium perenne*) and ~5-20% white clover (*Trifolium repens*) (Hoskin and Gee, 2004)), sourced from two 2 ha paddocks on an adjacent dairy farm (No. 4 Dairy Farm, Massey University, Palmerston North, New Zealand). Prior to Day 1 of the trial, the pasture had not been grazed for a 6 to 8-week period (previously grazed by cattle and never by horses) and had an average sward height of 15-20 cm (~3000-3500 kg DM/ha, Farmax sward stick, Hamilton, New Zealand). The grass was cut using a sickle bar mower (S.E.P.-BC 90, Reggio Emilia, Italy) to a height of 3-5 cm above the ground, between 0800-0900 hours (AM pasture cut) and 1600-1700 hours (PM pasture cut) each day (Appendix E-4). The cut grass was immediately transported to the trial site and stored in a feed room for less than 12 h before feeding.

The horses were provided with two hay nets containing ~10-15 kg and one bucket containing ~5-8 kg of cut pasture (fresh weight). As per individual horse requirements, the hay nets and feed buckets were refilled (either when empty or at ~4-h intervals - 0800, 1200, 1600, 2000 and 2400 h) to provide 1.3-2.3% BW of feed (DM basis). The quantity of cut pasture offered during Diet period P1 was restricted compared to *ad libitum* access to feed in diet periods P2 and P3.

During weeks 2, 4 and 6, one pair of horses were randomly allocated to one of the following diets, FE, FE+O or H+O according to a 3 × 3 Latin square design. Diet FE was a commercially available chopped ensiled forage diet containing HNF® High Nutritional Fiber (FiberEzy® [FE], Fiber Fresh Feeds Ltd., Reporoa, New Zealand), and comprised of lucerne (*Medicago sativa*; alfalfa; 50%) and timothy (*Phleum pratense;* 50%) grass, chopped into 1-5 cm stubbles, and ensiled with molasses (1%). The feed (FE) used during the trial was harvested as one batch (on 14th January 2014), and processed under similar conditions the next day. The lucerne forage was ~42 days of age with 10% flowering and harvested as a second cut of the season, and the timothy grass was fully mature, harvested as the first cut of the season. Both the grasses were ensiled and packaged in double plastic bags (22.7 kg net weight on as-fed basis). Diet FE was offered at a minimum of ~2.5-3.0% BW (DM basis) as two feeds at 0800 h and 2000 h, (~20 kg Diet FE/horse/day, on as-fed basis). To ensure *ad libitum* feeding, additional quantities of Diet FE were provided when less than 25% of the feed offered was remaining in the bucket.

Diet FE+O comprised of the same HNF® High Nutritional Fiber provided in Diet FE, mixed with whole oats (*Avena sativa*), and Diet H+O comprised of a perennial ryegrass hay fed with whole oats. The perennial ryegrass hay typically contained ~80% perennial ryegrass [*Lolium perenne*], and small proportions of white [*Trifolium repens*] and red [*Trifolium pratense*] clovers, herbs and weeds. The hay was harvested in January 2014, was processed as one batch at the same location (Manawatu, New Zealand), and was stored in a dry covered shed for ~6 months prior to the study period. Both the forage components of the diets FE+O and H+O were fed *ad libitum* at ~2% BW (DM basis)/horse/day.

The quantity of oats was calculated based on 50% of the minimum daily energy requirements for maintenance (DER_m) for a 500 kg horse (~35 MJ/horse/day) (Anonymous, 2007), equivalent to 2.5 kg DM/horse/day, divided into two feeds. Diet FE+O was provided in a feed bucket twice daily at 0800 and 2000 h, and to ensure *ad libitum* feeding, additional quantities of the chopped ensiled forage were provided when less than 25% of feed was remaining in the bucket. Hay was offered in two hay nets (weighing ~5-8 kg, as-fed basis) at 0800 h each day (similar to the procedure described for Diet P). The hay nets were replenished at 2000 h with additional quantities of hay, to provide *ad libitum* access to feed (Appendix E-3).

Refusals were collected twice daily (morning between 0700-0800 h and evening between 1900-2000 h) and weighed (TruTest-703 electronic scales, Auckland, New Zealand) to determine the amount of feed consumed (Glunk *et al.*, 2013b) (Appendix E-3).

7.3.6 Administration of indigestible markers

The passage rate of a diet (time taken for a diet to transit through the gastrointestinal tract) was estimated using solid-phase indigestible markers according to previously described methods (Blaxter *et al.*, 1956, McGreevy *et al.*, 2001, Pearson *et al.*, 2006, Rosenfeld *et al.*, 2006). The solid-phase markers used in the present trial were hollow cylindrical pieces (4-5 mm length, 5 mm outer diameter, ~40 mg weight) prepared from polyethylene tubing (Ledathene, Leda, Wellington, New Zealand). At 0800 h on the first day of each treatment block (Days 1, 8, 15, 22, 29 and 36), the horses were intubated by a veterinarian and 200 polyethylene markers were administered *via* a nasogastric tube (19 mm outer diameter × 3 m length, Kalayjian equine stomach tube, Shoof International Ltd., Cambridge, New Zealand), with 1-2 L of water. Green and blue coloured polyethylene markers were used on alternate 7 d treatment blocks (Appendix E-5). Two horses required mild sedation with 150 mg of

xylazine (Bomazine, Bomac Laboratories Ltd., Auckland, New Zealand) administered intravenously prior to nasogastric intubation. The total number of markers administered was recorded after correcting for any markers lost during intubation, and this value was used as the denominator to calculate the percentage of markers retrieved.

7.3.7 Data recording, sample collection and analysis

7.3.7.1 Body weight and condition

The general health and appetite of the horses were observed daily. Body condition scores (BCS, 1-9 (Henneke *et al.*, 1983)) and BW (measured using walk-on scales, TruTest economy plus-700, Auckland, New Zealand, accuracy 0.5 kg) of the horses were recorded on the 1st, 4th and 7th day of each treatment block.

7.3.7.2 Behaviour monitoring

During the first four days (Days 1-4, 8-12, 15-18, 22-25, 29-32 and 36-39) of each treatment block, the frequency of voiding faeces was recorded and the behaviour of horses was monitored by using an instantaneous scan sampling technique (Bateson, 1991), using the ethogram described in Table 7.2. Every hour, the behaviour of the horses was observed from a distance of 10-20 m outside the loose-box and one scan sample per horse was recorded (n=96 per horse per week, total N=3,456 observations). If an hourly observation was not recorded, it was considered as a missing value for all six horses at that time point.

Table 7.2. Ethogram describing horse behaviours observed during the study.

Behaviour Recorded	Description	References
Eating / drinking	Horse is actively chewing and swallowing food/edible material. Drinking from the water bucket.	McAfee *et al.* (2002) Winskill *et al.* (1996)
Standing alert	Rigid stance, with the neck elevated and the head orientated toward the object of focus. The ears are held stiffly upright and forward and the nostrils might be slightly dilated.	McDonnell and Haviland (1995) McDonnell (2003)
Resting (Standing or lying down)	Eyelid and lower lip drooped, neck at lowered angle ~ 30° to spine. Lying with sternum in contact with ground surface, legs folded under the body or stretched out on side with legs stretched out.	McBride and Cuddeford (2001) Young *et al.* (2012)
Abnormal behaviours	Crib-biting: Horse grips onto a fixed object using its incisor teeth, leans back onto hindquarters and contracts the strap muscle (contraction must be visible) of the neck to bring the head into an arched position. Air is sometimes taken into the oesophagus to produce a grunting sound. Weaving: Lateral movement of the head and neck from side to side in a rhythmic repetitive manner with alternation of the weight onto the contralateral foreleg with respect to the position of the head. Oral stereotypy: Repetitive oral activities without overt nutritional function, such as sham chewing, tongue rolling, biting or grasping stable fittings or repeated licking of pen fittings. Box-walking: Repeatedly moving between two locations in pen or walking a route around the stable.	McBride and Cuddeford (2001) McGreevy *et al.* (1995) Wickens and Heleski (2010) Cooper *et al.* (2005)
Other behaviours	Grooming, scratching, pawing, kicking, snorting, defecation/ urination, yawn, repetitive head movements	Young *et al.* (2012) McDonnell and Poulin (2002) McDonnell (2003)

7.3.7.3 Feed samples

Representative feed samples of each diet fed to the horses were collected to determine the nutrient content. One pooled sample of Diet P1 (n=7), and two samples (representing the AM and PM cuts) for Diets P2 (n=14) and P3 (n=14) were collected each day. One sample was collected on alternate days for the chopped ensiled forage used in Diets FE and FE+O (n=9) and hay used in Diet H+O (n=9), and one sample of whole oats was collected per week (n=3). The feed samples were weighed and stored at -20°C until analysis.

At the end of the trial, the feed samples (n=56) were lyophilised (FD18, Cuddon Freeze Dry, Blenheim, New Zealand) and ground to pass through a 1 mm screen (Cyclotec 1093 Sample Mill, Foss, Hillerod, Denmark), and analysed for nutrient content by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY). The method of analysis used was a combination of near infrared and plasma spectrophotometer techniques (Equi-Tech) to analyse dry matter (DM), crude protein (CP), neutral and acid detergent fibres (NDF, ADF), lignin, water and ethanol soluble carbohydrates (WCS, ESC), starch, fat, ash and minerals (Anonymous, 2015).

7.3.7.4 Faecal samples

Faecal matter was collected from all horses at hourly intervals from 6 h after nasogastric administration of the markers, until 96 h post-administration of markers (PAM). The faeces were stored separately in polyethylene bags (for recovery of the markers by manual sifting) (Appendix E-6). The total weight of the faeces voided (kg FW/d) by each horse was recorded at the end of each day. Faeces collected from the horses after 96 h PAM (Days 5-7 in each week), were collected directly into bins and weighed at the end of each day.

The markers were retrieved manually by sifting through the faecal matter collected during the first four days of each week, and the number of markers recovered was recorded to estimate gastrointestinal transit time.

7.3.8 Data analysis

All data were entered into a custom designed database (Microsoft Access, version 2010, Microsoft Corp., Redmond, WA) and explored for inconsistencies or outliers.

7.3.8.1 Determination of feed intake

Dry matter intake (DMI, kg/d) of each diet was calculated as the difference between the weight of feed consumed and the weight of the refusals (including spillage) measured on an as-is basis, and the values were corrected to a DM basis for each feed. In addition to the nutrient content estimations derived from the laboratory analysis, other feed values were calculated as follows: Total carbohydrates (CHO) = 100 - (CP + fat + ash); non-fibre carbohydrates (NFC) or non-structural carbohydrates (NSC) = 100 - (CP + fat + ash + NDF); and total digestible nutrients (TDN) = CP + (fat × 2.25) + NDF + NSC (Pagan, 1998, Hall, 2003). Digestible energy (DE) was calculated by the equation DE (MJ/kg DM) = 255 + 3660 × TDN × 0.00418 (Pagan, 1998). The daily energy intake was calculated for each diet based on an average of the estimated DE for each diet.

7.3.8.2 Behaviour observations

The various types of behaviours recorded during the scan sampling observations were consolidated and re-categorised to present the most commonly observed behaviours (eating, standing alert and resting). The remaining behaviours that were observed at very low frequencies were grouped into 'other behaviours' (grooming, licking objects or premises, vocalising and non-repetitive oral or head movements) or into 'abnormal behaviours' (repetitive box/yard walking, crib biting and weaving) (Appendix E-7). The data are presented as count and percentage of the type of behaviours recorded.

7.3.8.3 Determination of gastrointestinal transit time

The percentage of markers recovered in the faeces of each horse was calculated by dividing the number of markers recovered by the number of markers administered (corrected for losses during administration). The transit time delay of the markers was measured as the time (h) PAM to the first appearance of a marker in the faeces (T_1) (Moore-Colyer *et al.*, 2003, Rosenfeld *et al.*, 2006). The mean retention time (MRT, h) of the markers in the gastrointestinal tract for each diet was calculated by an equation previously described for particulate phase MRT in horses (Faichney, 1975, Rodrigues *et al.*, 2012).

7.3.8.4 Statistical analysis

The data were analysed in STATA version 12.1 (Stata Corp., College Station, TX). The distribution of variables in the dataset was tested for normality using the Shapiro-Wilk

test, and the data are presented as mean ± SD for parametric data or median ± interquartile range (IQR) for non-parametric data.

Significant differences between groups (diets and horses) were determined using an analysis of variance (ANOVA) for parametric data followed by a post-hoc Bonferroni test (dry matter intake, quantity of faeces voided, transit time, and mean retention time), and the Kruskal-Wallis test for non-parametric data (body weight and percentage of time spent eating). Count-time data (cumulative recovery of markers and frequency of voiding faeces) were tested for significant effects of diet and horse using Kaplan-Meier survival functions and the Log-rank test (Mantel-Cox test). Results were considered significant at $P<0.05$ and values between $P<0.05$ and $P<0.10$ were considered to reflect trends.

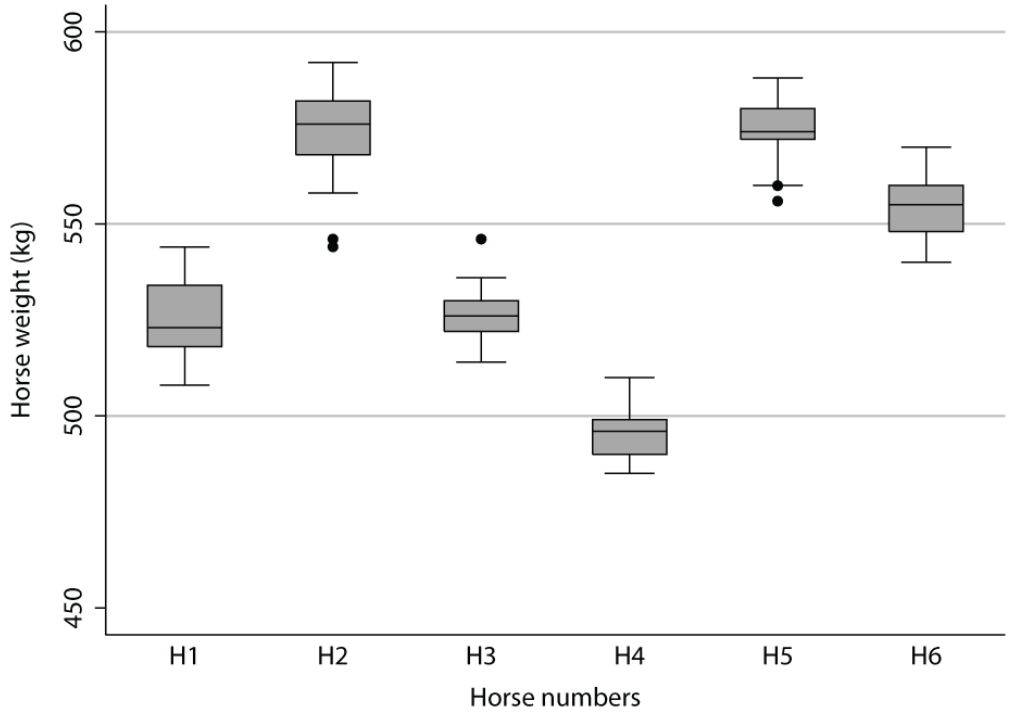
The DMI (g DM/kg $BW^{0.75}$) and the quantity of faeces voided (FW) by each horse on each diet, was calculated as average of the first four days in each dietary treatment block (n=36 observations), and the relationship of DMI and FW with MRT (h) were compared using the Spearman's rank correlation. The variables g DM/kg $BW^{0.75}$, kg FW and percentage of time spent eating (of 6 horses on 6 diets) were ranked as low or high, and MRT was ranked as short or long. The ranked variables were compared using Multiple Correspondence Analysis in two dimensions, with normalisation on principal coordinates.

7.4 Results

7.4.1 Body weight, condition and health

The horses remained clinically normal during the six-week study period. The faecal egg counts at the start of the study were between 0-50 eggs per gram of faeces. There was no significant change in body condition (BCS 5 throughout trial) or body weight during the study period (Figure 7.1, $P>0.05$) (Appendix E-8).

Figure 7.1. Distribution of the body weight of Thoroughbred horses measured three times weekly during the 6-week study period.



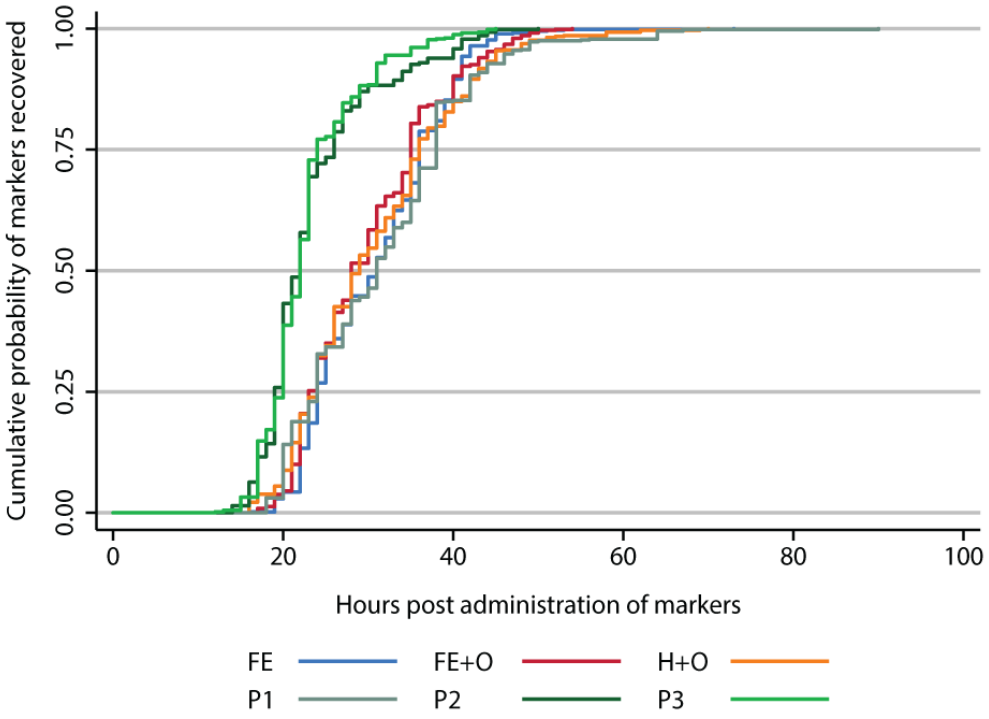
Legend: The box and whisker plots show the median body weight and interquartile range for the six horses, calculated from the pooled weights measured during the trial period (n=108 measurements). Within each horse, there was no significant difference in the body weight measure during the six-week study period (Kruskal-Wallis test P<0.05).

7.4.2 Marker recovery and gastrointestinal transit time

Overall, 75-95% of the markers administered to the horses were recovered from the faeces collected. The Kaplan-Meier survival functions indicated a significant effect of diet on the cumulative percentage of markers recovered in the faeces (P<0.001) (Figure 7.2). Diet P1 (restricted cut pasture) differed from diet periods P2 and P3 (*ad libitum* cut pasture) (P<0.001), but there was no difference between diet periods P2 and P3 (P=0.168). There was a trend for a difference between Diets FE, FE+O and H+O (P=0.061). There was a significant horse-effect on the cumulative percentage of markers recovered in the faeces (P<0.001) (Figure 7.3). There appeared to be greater variation in marker recovery curves for some diets (Diets P2 and

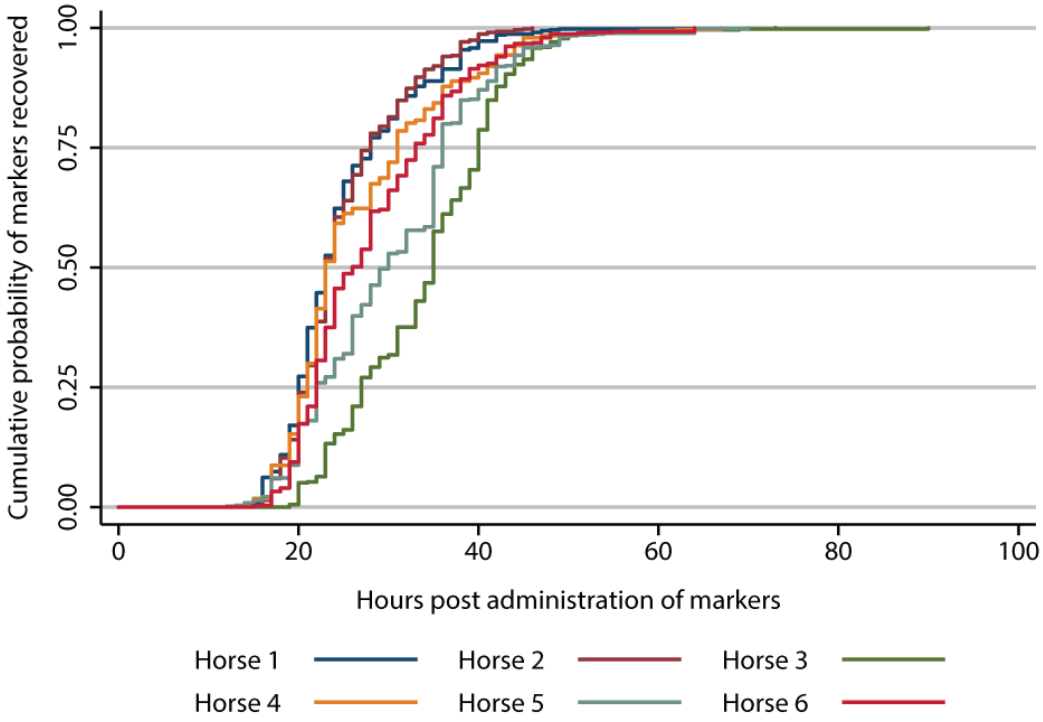
FE+O vs. Diet P3) and horses (H4, H5 and H6 vs. H2) (P<0.001 for each comparison, Appendix E-9, E-10 Additional file 1 and Appendix E-11 Additional file 2).

Figure 7.2. Comparison of the Kaplan – Meier survival functions for the cumulative percentage of polyethylene markers recovered in the faeces of six Thoroughbred horses fed six diets during the 6-week study period.



Legend: The diets comprised cut pasture (Diets P1, P2 and P3 fed in weeks 1, 3 and 5, respectively), an ensiled timothy-lucerne forage (Diet FE), ensiled timothy-lucerne forage fed with oats (Diet FE+O) and perennial ryegrass hay fed with oats (Diet H+O). The Log-rank test showed a significant effect of diet on the cummulative percentage of markers recovered (P<0.001), with no differences between Diets P2 and P3 (P=0.168), but significant differences between Diet P1 vs. Diets P2 and P3 (P<0.001) and a significant trend for differences between the Diets FE, FE+O and H+O (P=0.061).

Figure 7.3. Comparison of the Kaplan – Meier survival functions for the cumulative percentage of polyethylene markers recovered in the faeces of six Thoroughbred horses fed cut pasture or conserved forage-based diets during the 6-week study period.



Legend: The Log-rank test showed a significant horse-effect on the cumulative percentage of markers recovered ($P<0.001$).

The mean time in hours (h) ± SD for recovery of the 1st marker for each diet across all horses was 19 ± 4 (P1), 14 ± 3 (P2), 15 ± 3 (P3), 21 ± 5 (FE), 19 ± 4 (FE+O) and 17 ± 2 (H+O), and for each horse across all diets was 16 ± 4 (H1), 17 ± 2 (H2), 22 ± 5 (H3), 16 ± 3 (H4), 18 ± 4 (H5), and 15 ± 3 (H6). Both diet ($P<0.01$) and horse ($P<0.01$) had a significant effect on the time at which the 1st marker was recovered (adjusted $R^2=0.52$).

Retention time (h) of the markers in the gastrointestinal tract for each horse, stratified by diet, is presented in Table 7.3. There was a significant effect of diet ($P<0.05$) and horse ($P<0.001$) on the MRT of digesta (adjusted $R^2=0.87$).

Table 7.3. Mean retention time (h) of markers recovered in the faeces obtained from horses during the study.

	Dietary treatments						
	P1	**P2**	**P3**	**FE**	**FE+O**	**H+O**	**Mean ± SD**
H1	28	20	22	31	30	24	$26^{a} \pm 4$
H2	26	21	24	28	28	26	$26^{a} \pm 2$
H3	38	31	26	38	38	35	$34^{b} \pm 4$
H4	30	24	22	28	25	36	$28^{ab} \pm 5$
H5	38	25	23	36	33	32	$31^{ab} \pm 5$
H6	34	23	23	28	29	35	$29^{ab} \pm 5$
Mean ± SD	$32^{a} \pm 5$	$24^{b} \pm 4$	$23^{b} \pm 2$	$32^{a} \pm 4$	$31^{a} \pm 5$	$31^{a} \pm 5$	

Legend: Significant diet and horse effects ($P<0.05$ and $P<0.001$, respectively, $R^2=0.87$). Different superscripts within a row or column indicate significant differences (post-hoc Bonferroni test).SD - standard deviation; H1-6 - horse numbers; P1, P2 and P3 - cut pasture fed to the horses in weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage mixed with whole oats; H+O - perennial ryegrass hay fed with whole oats.

7.4.3 Faecal voiding

The horses voided between 8-21 kg/d of faeces (fresh weight) at the rate of one faecal output every 2 h. The quantity of faeces voided per day differed significantly between diets ($P<0.0001$) and between horses ($P<0.0001$) (adjusted $R^2=0.88$). Horses 1 and 2 voided a greater weight of faeces (12-21 kg/d) when compared to the other horses (8-16 kg/d) (Table 7.4). The Kaplan-Meier survival functions indicated a significant diet effect ($P=0.046$) on the cumulative frequency of faeces voided, with no difference observed between horses ($P=0.441$) (Appendix E-12, Additional file 3).

Table 7.4. Faeces voided by horses (kg/d) when fed each diet during the study period.

	Dietary treatments						
	P1	**P2**	**P3**	**FE**	**FE+O**	**H+O**	**Mean ± SD**
H1	12 ± 1	18 ± 5	19 ± 3	16 ± 4	19 ± 3	16 ± 3	17^{a} ± 4
H2	14 ± 3	20 ± 5	19 ± 3	19 ± 5	21 ± 4	14 ± 4	18^{a} ± 5
H3	8 ± 2	9 ± 2	13 ± 3	13 ± 3	9 ± 2	11 ± 3	11^{bc} ± 3
H4	8 ± 2	14 ± 3	14 ± 3	10 ± 2	10 ± 3	11 ± 2	11^{cd} ± 3
H5	9 ± 3	14 ± 3	16 ± 2	16 ± 4	12 ± 2	12 ± 2	13^{d} ± 4
H6	10 ± 1	14 ± 4	16 ± 3	16 ± 4	13 ± 4	9 ± 1	13^{d} ± 4
Mean ± SD	10^{a} ± 3	15^{b} ± 5	16^{b} ± 3	15^{b} ± 5	14^{bc} ± 5	12^{ac} ± 3	

Legend: Significant diet and horse effects ($P<0.0001$ and $P<0.0001$, respectively, $R^2=0.88$). Different superscripts within a row or column indicate significant differences (post-hoc Bonferroni test). SD- standard deviation; H1-6 - horse numbers; P1, P2 and P3 - cut pasture fed to the horses in weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage mixed with whole oats; H+O - perennial ryegrass hay fed with whole oats.

7.4.4 Feed intake

All horses consumed between 1.1-1.5% BW of a feed (DM basis), equivalent to 64-91 MJ of digestible energy per day. The quantity of feed offered to, and consumed by, the horses is given in Table 7.5. The DMI (kg/d) differed significantly between horses ($P<0.0001$) and diets ($P<0.0001$), with a significant diet × horse interaction ($P<0.0001$) adjusted $R^2=0.95$. The between horse effect remained significant when DMI was expressed as %BW and $BW^{0.75}$ ($P<0.0001$ for each comparison). Within each dietary treatment week, the horses consumed ~1-5% less feed on the first day than the subsequent days in the week, but this reduction in feed intake was not significant. The quantity of feed (g DM/kg $BW^{0.75}$/d) consumed was highest for the diets containing chopped ensiled forage (Diets FE and FE+O), followed by the Diets P2, P3 and H+O, and lowest for Diet P1. Across all diets, horses 1 and 2 consumed higher quantities of feed than the other horses (Table 7.6).

Table 7.5. Feed offered and consumed by the horses during the study period.

		Dietary treatments					
	Units	**P1**	**P2**	**P3**	**FE**	**FE+O**	**H+O**
Feed offered	kg/d (as-fed basis)	46 ± 10	57 ± 8	60 ± 9	24 ± 4	27 ± 5	15 ± 3
Feed consumed	kg/d (as-fed basis)	38 ± 8	44 ± 8	46 ± 9	19 ± 4	19 ± 5	9 ± 2
Feed consumed	kg/d (dry matter basis)	6.1 ± 1.3	7.0 ± 1.3	7.3 ± 1.4	7.4 ± 1.5	7.6 ± 2.1	6.9 ± 1.5

Legend: P1, P2 and P3 - cut pasture fed to the horses in weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage mixed with whole oats; H+O - perennial ryegrass hay fed with whole oats.

Table 7.6. Feed (g DM/kg $BW^{0.75}$/d) consumed by horses during the study period.

	Dietary treatments						
	P1	**P2**	**P3**	**FE**	**FE+O**	**H+O**	**Mean ± SD**
H1	62 ± 8	71 ± 10	78 ± 10	64 ± 8	91 ± 12	73 ± 19	73^{a} ± 15
H2	63 ± 11	70 ± 8	72 ± 8	68 ± 4	87 ± 11	65 ± 15	70^{a} ± 12
H3	50 ± 12	50 ± 7	56 ± 10	61 ± 10	45 ± 4	56 ± 10	53^{c} ± 10
H4	47 ± 9	54 ± 7	60 ± 11	58 ± 10	54 ± 5	53 ± 5	54^{cd} ± 9
H5	49 ± 10	62 ± 6	64 ± 7	64 ± 13	61 ± 3	63 ± 7	61^{bd} ± 10
H6	54 ± 9	62 ± 7	64 ± 12	80 ± 15	74 ± 3	61 ± 5	66^{ab} ± 12
Mean ± SD	54^{e} ± 11	62^{f} ± 11	65^{f} ± 12	66^{f} ± 12	69^{f} ± 19	62^{f} ± 13	

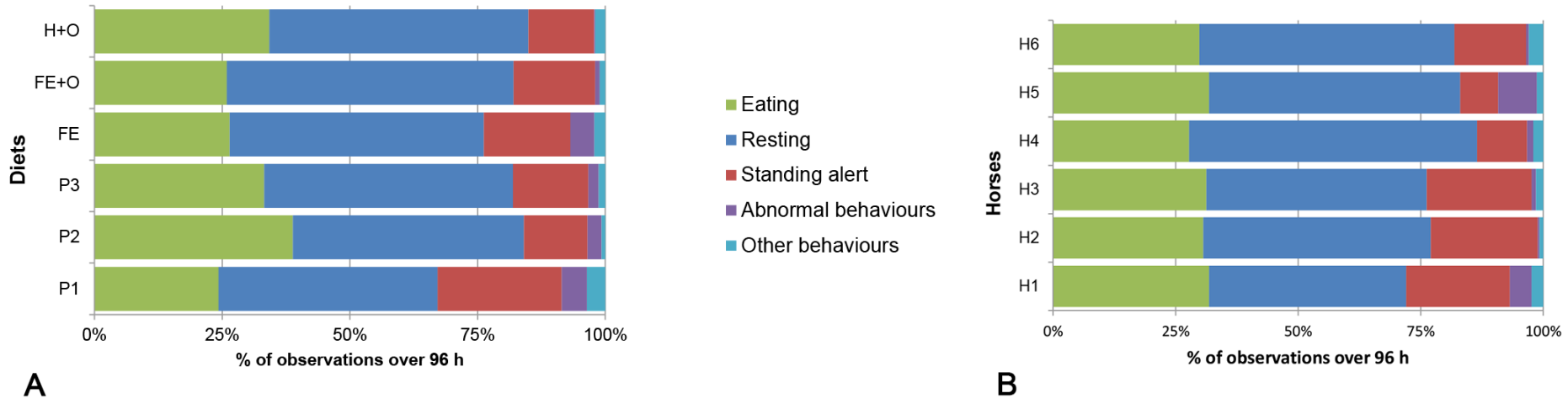
Legend: The kg of DM consumed differed significantly between horses ($P<0.0001$) and diets ($P<0.0001$), with diet × horse interaction, $R^2=0.95$. Different superscripts within a row or column indicate significant differences (Bonferroni test). DM - dry matter; SD - standard deviation; H1-6 - horse numbers; P1, P2 and P3 - cut pasture fed to the horses in weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage mixed with whole oats; H+O - perennial ryegrass hay fed with whole oats.

7.4.5 Behaviour monitoring

When observed at hourly-intervals over the first four days of each treatment block, horses were most often found to be resting (43-56% of total observations), eating (24-39%) or standing alert (12-24%), with a similar pattern observed across all diets (Figure 7.4). Other behaviours (grooming, licking objects or premises, vocalising and non-repetitive oral or head movements) and abnormal behaviours (box or yard walking, weaving, crib biting) were observed <5% of the time. Most of the abnormal behaviours were observed in two horses (H1 - box walking and H5 - weaving) at a low frequency (4% and 8%, respectively) (Figure 7.4).

There was a significant difference in the percentage of time spent eating between diets ($P<0.001$), with no significant differences between horses ($P=0.96$) (Figure 7.4). Horses were observed to be eating more often during diet periods P2 and P3, when compared to diet period P1 ($P<0.01$) and this finding was consistent with the amount of feed offered during the diet periods. Horses spent more time eating Diet H+O than Diets FE and FE+O ($P<0.01$, Figure 7.4). Some diets (Diets P1, FE and FE+O) appeared to show more variation between horses (Appendix E-13, Additional file 4).

Figure 7.4. Behaviour of horses recorded over a 96-hour period within each treatment block of the study.

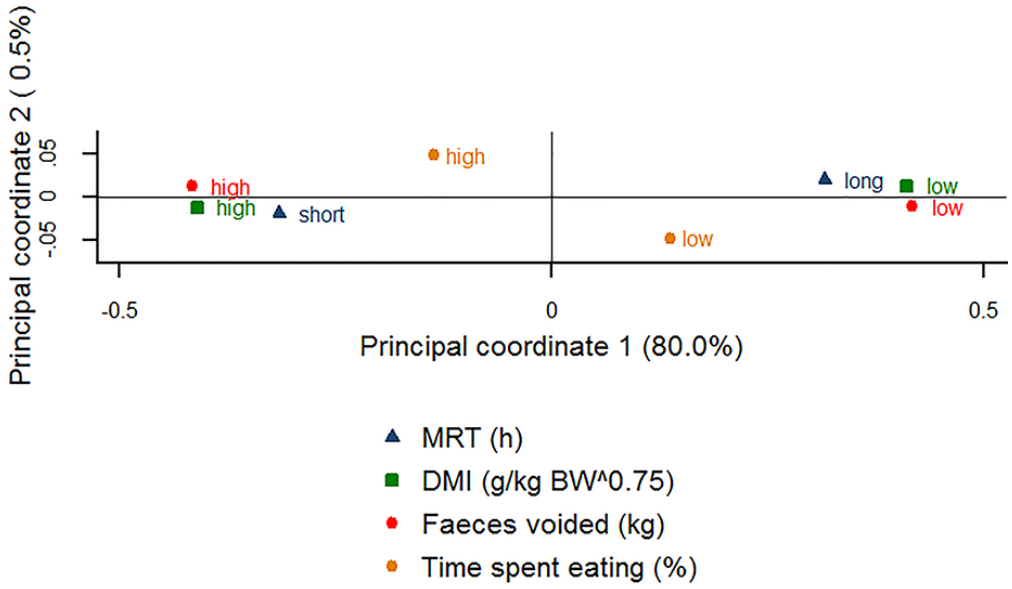


Legend: The stacked bar charts indicate the behaviour observed as percentage of the total number of observations during each 96-hour period. Panel A shows the distribution of the behaviour exhibited by the six horses, stratified by diet. There was a significant difference in the eating behaviour of horses when compared between diet groups ($P<0.001$). Panel B shows the distribution of the behaviour exhibited by each horse across all diets. There was no significant differences observed between the six horses when compared across diets ($P=0.96$). The diets fed to the horses were cut pasture (P1, P2 and P3, fed in weeks 1, 3 and 5, respectively), a commercial chopped ensiled forage mixed with whole oats (Diet FE+O) or without whole oats (Diet FE), and ryegrass-clover hay fed with whole oats (H+O). The 'abnormal behaviours' category included grooming, licking objects or premises, vocalising and non-repetitive oral or head movements and the 'other behaviours' category included repetitive box/ yard walking, crib biting and weaving.

7.4.6 Multivariate comparisons

Mean retention time was negatively correlated with kg DMI/d (R=-0.45), g DM/kg $BW^{0.75}$ (R= -0.51) and kg FW (R= -0.64). Multiple correspondence analysis showed that horses that consumed more feed and voided more faeces, had a shorter MRT when compared to horses that consumed less feed and voided less faeces, with 80% of the variation explained on one principal coordinate (Figure 7.5).

Figure 7.5. Multiple correspondence analysis of mean retention time, dry matter feed intake and quantity of faeces voided in horses involved in the study.



Legend: MRT – mean retention time of digesta in the gastrointestinal tract; DMI – dry matter intake of the feed; Faeces voided was measured as fresh weight (kg).

7.5 Discussion

The present study investigated the effects of abrupt dietary transition on the passage rate of digesta through the gastrointestinal tract, and the results obtained supported the hypothesis that diet and horse have significant effects on the passage rate of digesta. The proportion of polyethylene markers recovered in the faeces was equal to, or higher than, the recovery rates reported in other studies (McGreevy *et al.*, 2001, Boscan *et al.*, 2006). This high percentage of marker recovery and the frequency of faecal collection (hourly intervals)

indicated that a relatively high precision could be attained when estimating the MRT of the diets used in the present study (Rosenfeld *et al.*, 2006).

The feed samples were analysed using near infrared and plasma spectrophotometer techniques, and hence, the nutrient values presented in this study may not be directly comparable with other published studies using wet chemistry. Another potential limitation in the methodology of the study was the collection of spillage from Diets FE and FE+O, which was difficult to retrieve from the sawdust bedding. However, the values were corrected using a visual estimate of the percentage of sawdust mixed with the spilled diet.

The abrupt dietary transitions may have affected the feed intake of the horses in the present study, as small decreases in DMI were observed on the days the diets were changed. However, irrespective of the diet offered, the average DMI did not decrease below 1.3% BW, indicating that the horses consumed sufficient DM (~7 kg DM/horse/d). The DE (~64-91 MJ/d) obtained from each feed was within the NRC recommended guidelines for maintenance (Anonymous, 2007), as reflected by the constant BW of all horses throughout the study period.

Only two horses (H1 and H5) exhibited abnormal behaviours (box-walking and weaving, respectively), which were observed rarely, irrespective of diet. Weaving and box walking are typically expressed in anticipation of more (new) food, or due to confinement in stables (Cooper *et al.*, 2005), which appeared to be the situation with horses 1 and 5. Despite the abnormal behaviours, the time spent eating for horses 1 and 5 did not differ from that of the other horses and their body weight remained constant during the study.

Shorter MRTs have been reported after feeding *ad libitum* versus restricted quantities of alfalfa and oat straw (Pearson *et al.*, 2001). A similar reduction in T_1 and MRT was observed in the present study when horses were fed *ad libitum* (P2 and P3) compared to restricted (P1) quantities of cut pasture. The MRT of digesta increased from 23-24 h to 32 h when the level of cut pasture was restricted, and became similar to the MRTs observed when the horses were fed *ad libitum* conserved forage (31-32 h). The adaptation of horses to the inside stable environment during Week 1 may have confounded the results of the study, but the clustering of marker-recovery curves confirmed that the level of feeding (*ad libitum* vs. restricted), and hence feed intake, had a significant effect on, and perhaps drives the passage rate of digesta through the gastrointestinal tract.

The MRTs of digesta were consistent between the periods when the horses were fed *ad libitum* cut pasture (P2 and P3), and these values were similar to a previous report of weanling Thoroughbred horses fed cut pasture in stables or when grazing *ad libitum* in paddocks (Grace *et al.*, 2003). The comparable MRTs reported between the studies is noteworthy, considering the differences in the age of horses (adult vs. weanling) and the type of particulate phase markers (polyethylene markers vs. mobile nylon bag technique) used. However, it may be difficult to compare our results with the MRTs obtained from previous studies due to differences in the methodologies used. For example, the MRTs observed for Diet FE (32 ± 4 h) in the present study appeared to be within the range previously reported for chopped ensiled forage (27-29 h), but the T_1 for the Diet FE (21 ± 5 h) was significantly longer than for the previously reported chopped ensiled forage (11 h) (Moore-Colyer *et al.*, 2003). The differences observed between the studies may be due to differences in the type of particulate phase markers used (polyethylene markers vs. Ytterbium-labelled forage) and the different algorithms used to calculate the MRTs, or because the present study was conducted on Thoroughbred horses, and not Welsh-cross ponies. When compared on a per kg metabolic body weight, the Welsh-cross pony geldings that were fed at restricted levels, consumed similar quantities of ensiled forage (62 vs. 66 g DM/kg $BW^{0.75}$/d) as the Thoroughbred mares fed *ad libitum* in the present study, indicating that the rate of feed intake may have been slower in our horses compared to the ponies. Therefore, given the variability in the feed intake of different equid types (e.g. horses, ponies and donkeys) (Cuddeford *et al.*, 1995, Drogoul *et al.*, 2001, Pearson *et al.*, 2001), some caution is warranted when comparing the results obtained in the present study with previous investigations, the majority of which were conducted on ponies (Cuddeford *et al.*, 1995, Pearson *et al.*, 2001, Moore-Colyer *et al.*, 2003).

The inconsistencies in the feed intake and passage rate of digesta reported in previous work may have been due to differences in the feeding and management of the animals used in the studies (de Fombelle *et al.*, 2004, Rodrigues *et al.*, 2012, Jensen *et al.*, 2014). A large variation in feed intake and passage rate of digesta was also observed between the horses in the present study, even though the experimental design controlled for breed, diet and management of the horses. The MRT varied by up to 10 h between the horses, with the longest MRT observed for Horse 3, and this slower passage rate appeared to be linearly related to the DM of feed consumed (Horse 3 consistently consumed less DM than other horses in the study). Furthermore, the DMI varied between the horses for Diet FE+O (45-91 g DM/kg $BW^{0.75}$) and Diet H+O (53-73 g DM /kg $BW^{0.75}$), and this variation in DMI was most likely due to the

differences in the DMI for the roughage component of the diets (chopped ensiled forage and hay), because the grain component (oats) was completely consumed by all horses (Morel, 2010). The horses consumed less chopped ensiled forage when fed on Diet FE+O than Diet FE (~46 g vs. 66 g DM/kg $BW^{0.75}$/d), possibly due to the substitution effect of grain supplements incorporated in the diet (Stowers *et al.*, 2009), and the likelihood that horses regulated their feed intake depending on the energy content in the diet (Laut *et al.*, 1985). Perhaps other horse-specific factors, such as the rate of DMI, individual metabolic rates and reproductive status, also influence the feeding behaviour of horses, but the effects of these factors on the DMI and passage rate of digesta are poorly understood, and may require further investigation.

The model for DMI (g DM/kg $BW^{0.75}$/d) showed a significant diet × horse interaction, such that some horses were observed eating Diets FE and FE+O more frequently than Diet H+O. Horses prefer forages harvested at early maturities (Staniar *et al.*, 2010, Särkijärvi *et al.*, 2012), and preferentially consume greater quantities of lucerne forage when compared to other forage species (LaCasha *et al.*, 1999, Rodiek and Jones, 2012), and ensiled forage when compared to hay prepared from the same forage species (Müller and Udén, 2007). The preference of some horses for diets containing ensiled forage (45% moisture) when compared to hay (10% moisture), may be due to the greater palatability of the chopped ensiled forage diet and the shorter time required to consume it (chopped forage of low DM content takes less time to chew and mix with saliva than long-stem forage of a higher DM content) (Müller and Udén, 2007). Differences in the fibre length of the forages (cut pasture, chopped ensiled forage and long-stem hay) may have had some influence on the MRTs of digesta (Moore-Colyer *et al.*, 2003), but the effects of diet on the passage rate of digesta observed in the present study appeared to be more likely due to differences in other horse-specific factors that may have influenced the feed intake levels, such as individual preferences or palatability of a diet. Nonetheless, the rate of adaptability to a diet, its palatability and individual preferences of horses are poorly understood and merits further investigation.

Some horses in the present study had higher DMIs and shorter MRTs than others. The underlying reason for the inter-horse variation, may be similar to that observed for an inter-species variation, where donkeys were found to consume less DM than ponies, and consequently had longer retention times and higher apparent digestibility of the feed consumed (Cuddeford *et al.*, 1995, Pearson *et al.*, 2001, Boscan *et al.*, 2006). Dry matter intake is negatively correlated with the apparent digestibility of feed (Morel, 2010), and this

relationship supports the argument that longer retention time allows more time for microbial fermentation in the hindgut and increases the apparent digestibility of the feed consumed (Cuddeford *et al.*, 1995). This physiological strategy may be beneficial to the survival of horses under feral conditions, where decreases in growth rates, and thus pasture availability in summer and winter, may reduce the quantity of feed consumed, but a consequent increase in the MRT of digesta may allow better digestibility of the feed consumed, which may then meet the daily energy requirements for maintaining body weight (Kuntz *et al.*, 2006). However, this potential survival strategy could be a problem when managing obese domestic horses and ponies (easy-keepers), including those predisposed to pasture-associated laminitis, when kept on restricted access to pasture (shorter grazing turnout periods or limited sward height) designed to reduce their caloric intake (Geor, 2010). Horses and ponies are capable of increasing their forage consumption during restricted grazing periods by increasing the rate of feed intake, with reports of ~40% of daily DM intake consumed by ponies during a 3-h restricted grazing turnout (Ince *et al.*, 2005), and greater mean rates of DM intake observed in horses during 3- and 6-h restricted grazing turnouts than 24-h access to grazing (Glunk *et al.*, 2013a) . Although restricting pasture access decreases the total intake of pasture, the compensatory increase in the rate of feed intake (during the turnout period) and the longer MRT of digesta (due to the restricted feeding time) may enable horses and ponies to maintain BW even when the quantity of feed is restricted (Dugdale *et al.*, 2011, Argo *et al.*, 2012, Glunk *et al.*, 2013a).

Since variation in feed intake affects the passage rate of digesta and the apparent digestibility, these factors should be taken into consideration when formulating specific feeding regimens for horses and ponies, and when comparing the rate of change in microbiota populations when horses transition between diets. The differences observed in the feed intake levels and MRTs may explain some of the previously reported variation in the population of hindgut or faecal microbiota (Dougal *et al.*, 2014, Fernandes *et al.*, 2014), and perhaps explains the increased susceptibility of some horses and ponies to gastrointestinal disturbances. This hypothesis requires further investigation.

7.6 Conclusion

The results of the present study highlighted that diet-specific factors (such as feed type, composition and intake level) could influence the MRT of digesta in the gastrointestinal

tract. As expected, there was large variation between the horses due to horse-specific factors (such as eating behaviour, dry matter intake and quantity of faeces voided). It was concluded that the level of feed intake drives the passage rate of digesta, and thus, may potentially increase the apparent digestibility of the feed consumed, due to the greater time available for microbial fermentation.

7.7 Acknowledgements

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Appendix E

This appendix provides supplementary information on the tables and figures cited in Chapter 7.

E-1. Figure illustrating the experimental design of the feeding trial including six Thoroughbred horses.

E-2. Layout of the trial site and management of the horses.

E-3. Diets, feeding management and collection of refusals.

E-4. Cutting and collection of pasture to feed horses during weeks 1, 3, and 5 of the study period.

E-5. Administration of polyethylene markers, collection of faeces and retrieval of markers from faeces.

E-6. Collection of faeces and retrieval of polyethylene markers.

E-7. Example of behaviours described in the ethogram and observed during scan sampling of the six horses over the study period.

E-8. Body weight of the horses measured on Days 1, 4 and 7of each treatment block.

E-9. Time (h) for recovery of markers in the faeces of horses included in the study.

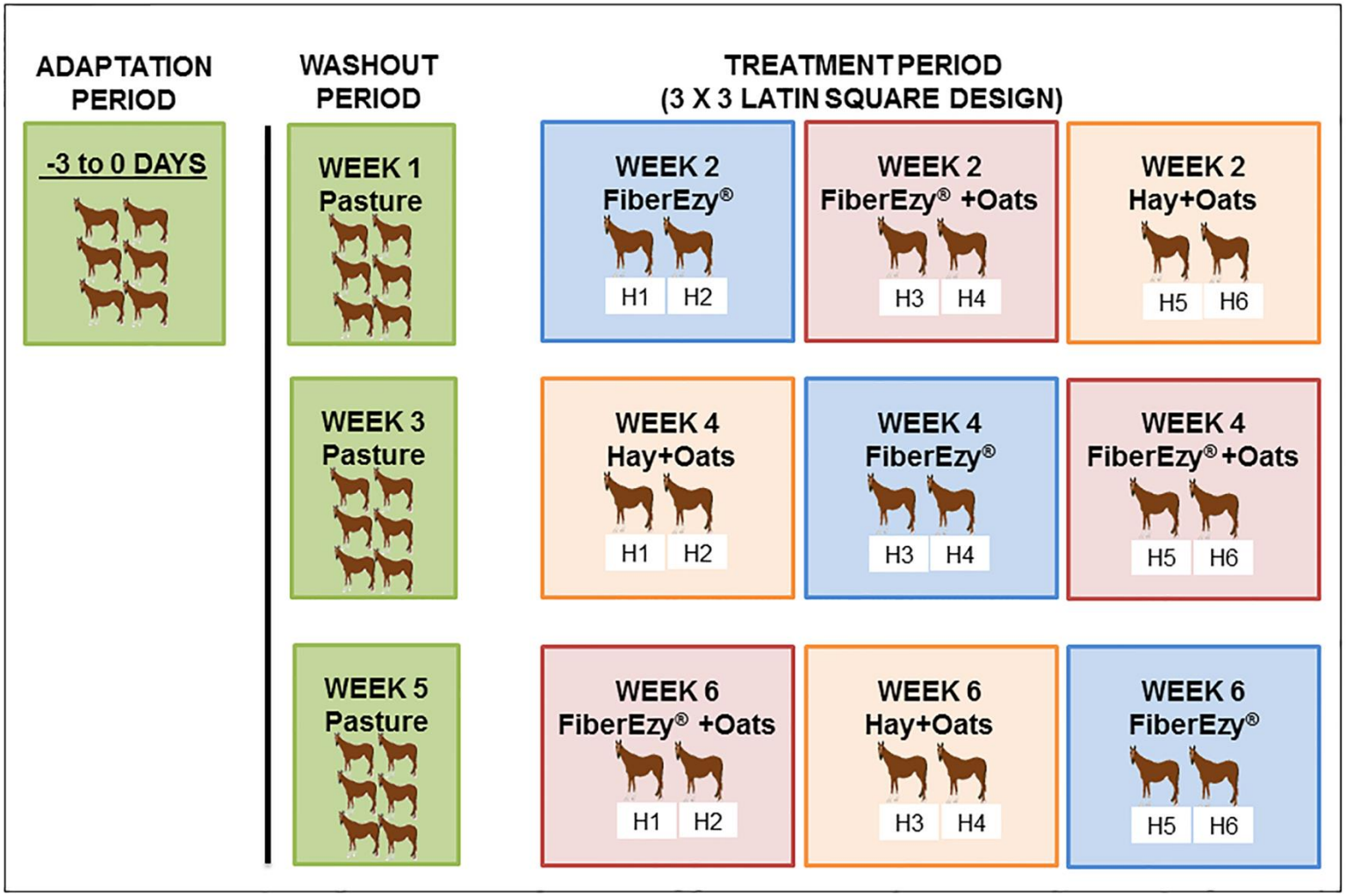
E-10. Additional file 1. Kaplan – Meier survival analysis of the cumulative percentage of markers recovered in the faeces of the six horses, on each diet fed during the six-week study period.

E-11. Additional file 2. Kaplan – Meier survival analysis of the cumulative percentage of markers recovered in the faeces of the six horses, stratified by horse across diets fed during the six-week study period.

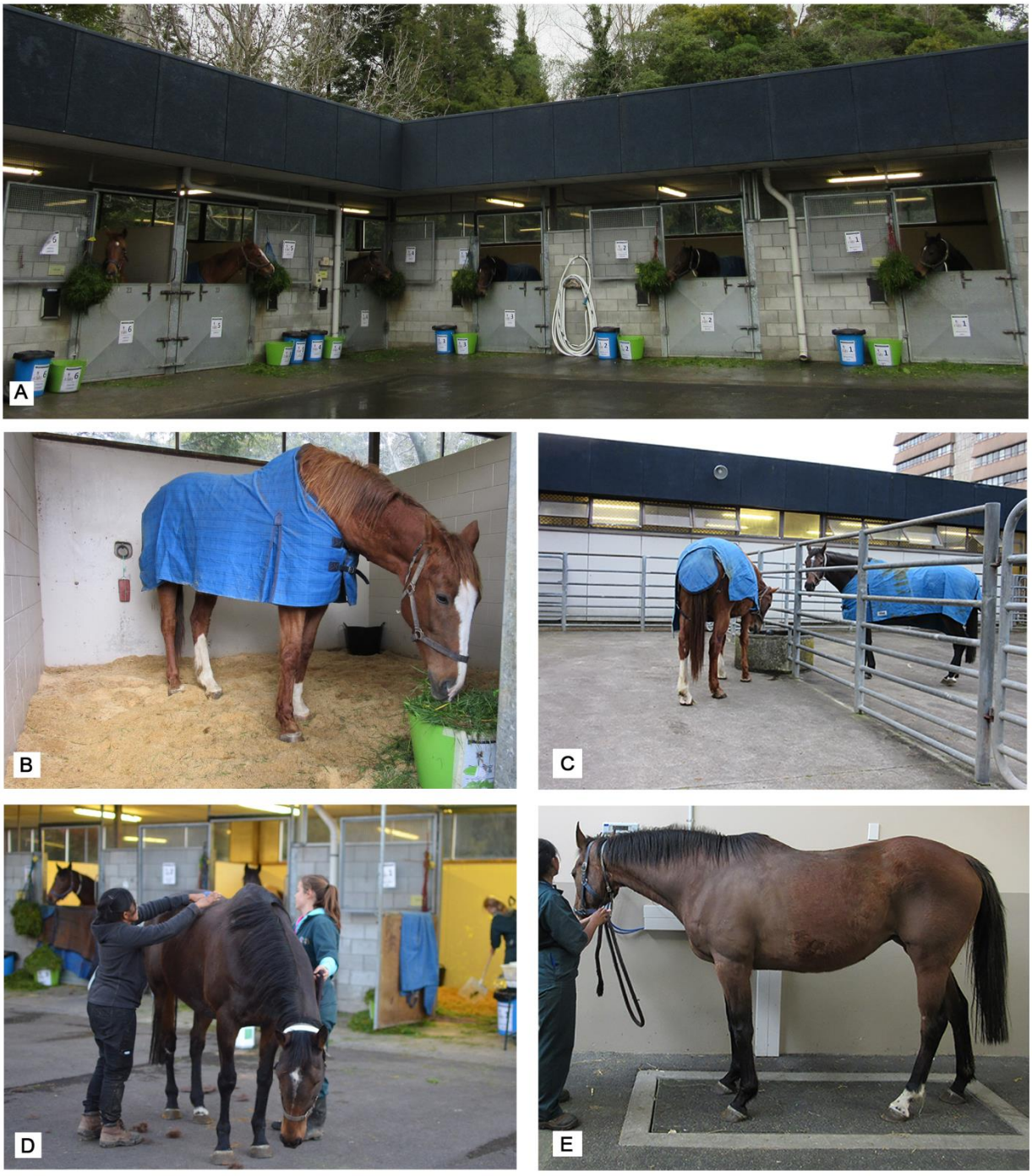
E-12. Additional file 3. Kaplan – Meier survival analysis of the cumulative frequency of faeces voided during the first four days of each dietary treatment block, stratified by horse and diet.

E-13. Additional file 4. Behaviour of horses recorded over a 96-hour period, compared within diets fed during the study.

E-1. Figure illustrating the experimental design of the feeding trial including six Thoroughbred horses.



E-2. Layout of the trial site and management of the horses.



A) Layout of the trial site.

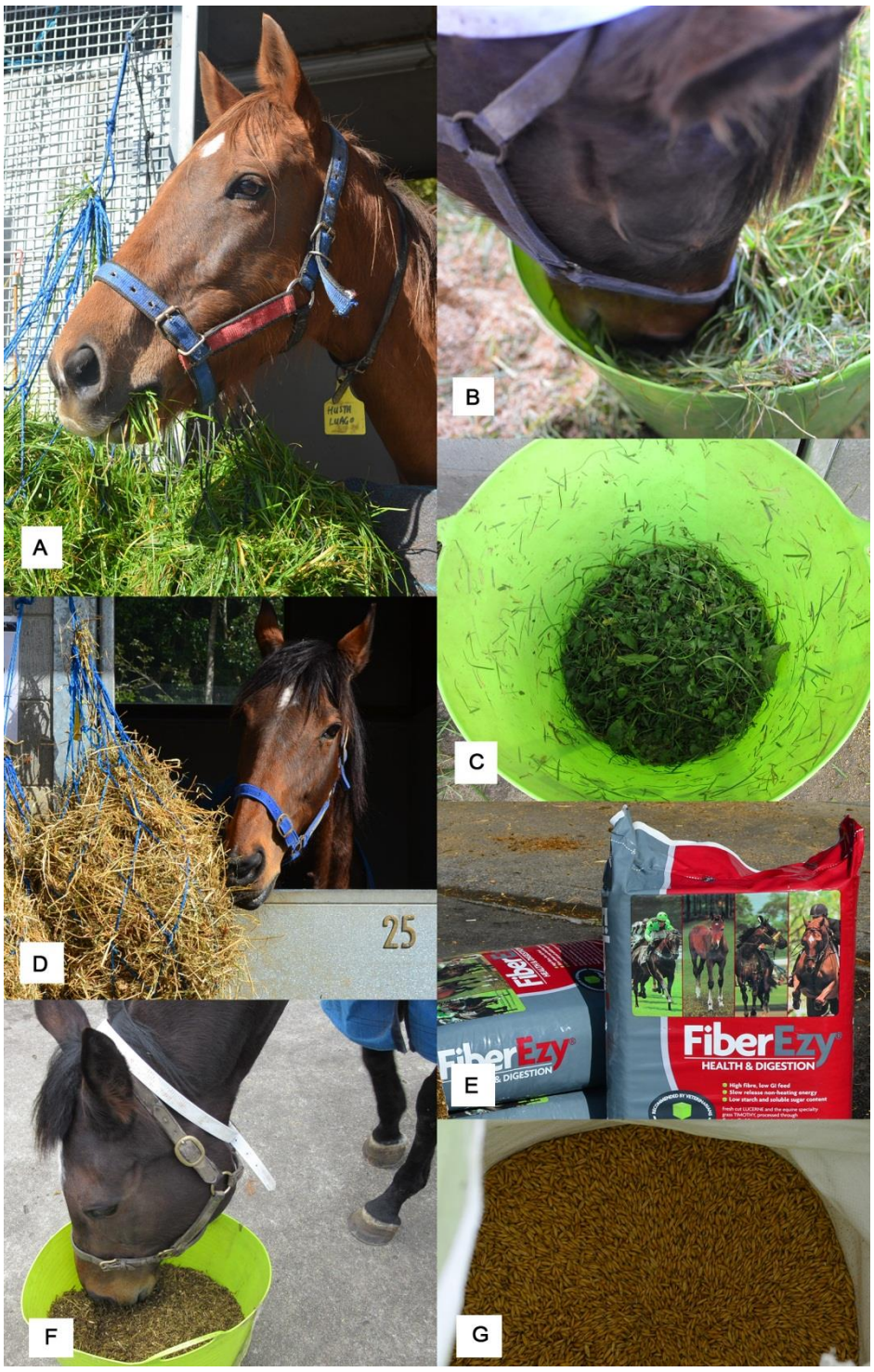
B) Layout of a loose-box, including feeding and water buckets, salt block and bedding.

C) Layout of the yards in which the horses were turned out twice daily.

D) Grooming and stable management for the horses.

E) Measuring body weight of the horses on walk-on electronic scales.

E-3. Diets, feeding management and collection of refusals.



A) Pasture fed in a net.

B) Pasture fed in a feed bucket.

C) Refusal collected in a feed bucket.

D) Hay fed in a net.

E) FiberEzy® forage diet.

F) FiberEzy® fed in a feed bucket.

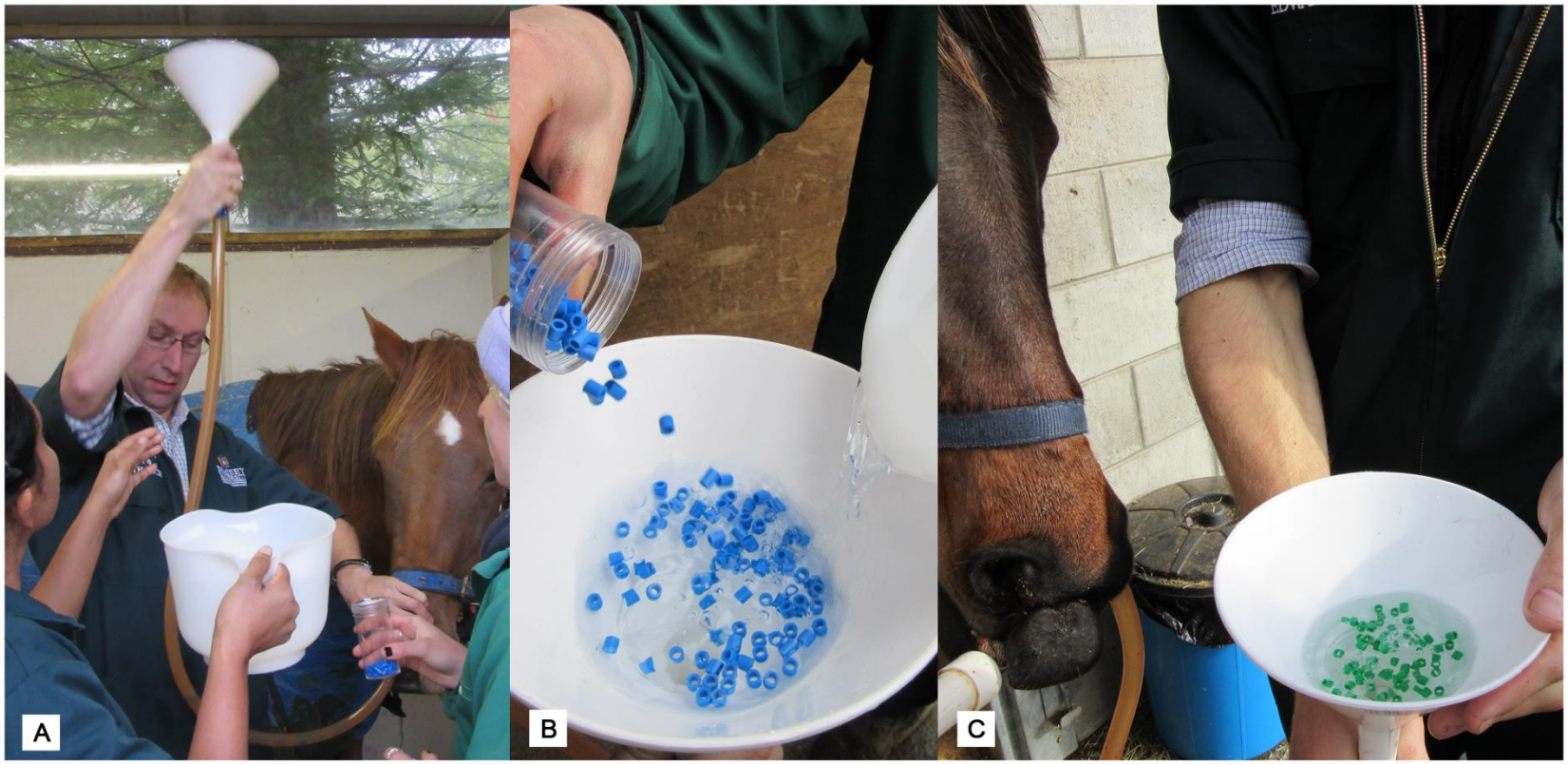
G) Whole oats offered to the horses.

E-4. Cutting and collection of pasture to feed horses during weeks 1, 3, and 5 of the study period.

A) Sickle bar mower and weedeater used to cut the grass at grazing height from the ground.

B) Collection of cut pasture.

E-5. Administration of polyethylene markers via a nasogastric intubation of the horses during the study.



A) Nasogastric intubation for administration of polyethylene markers.

B) Blue polyethylene markers administered during weeks 2, 4 and 6 of the trial.

C) Green polyethylene markers administered during weeks 1, 3 and 5 of the trial.

E-6. Collection of faeces and retrieval of polyethylene markers.

A) Collection of faeces. B) Spotting indigestible markers voided in the faeces.

C) Manual sifting of faecal matter for retrieval of polyethylene markers.

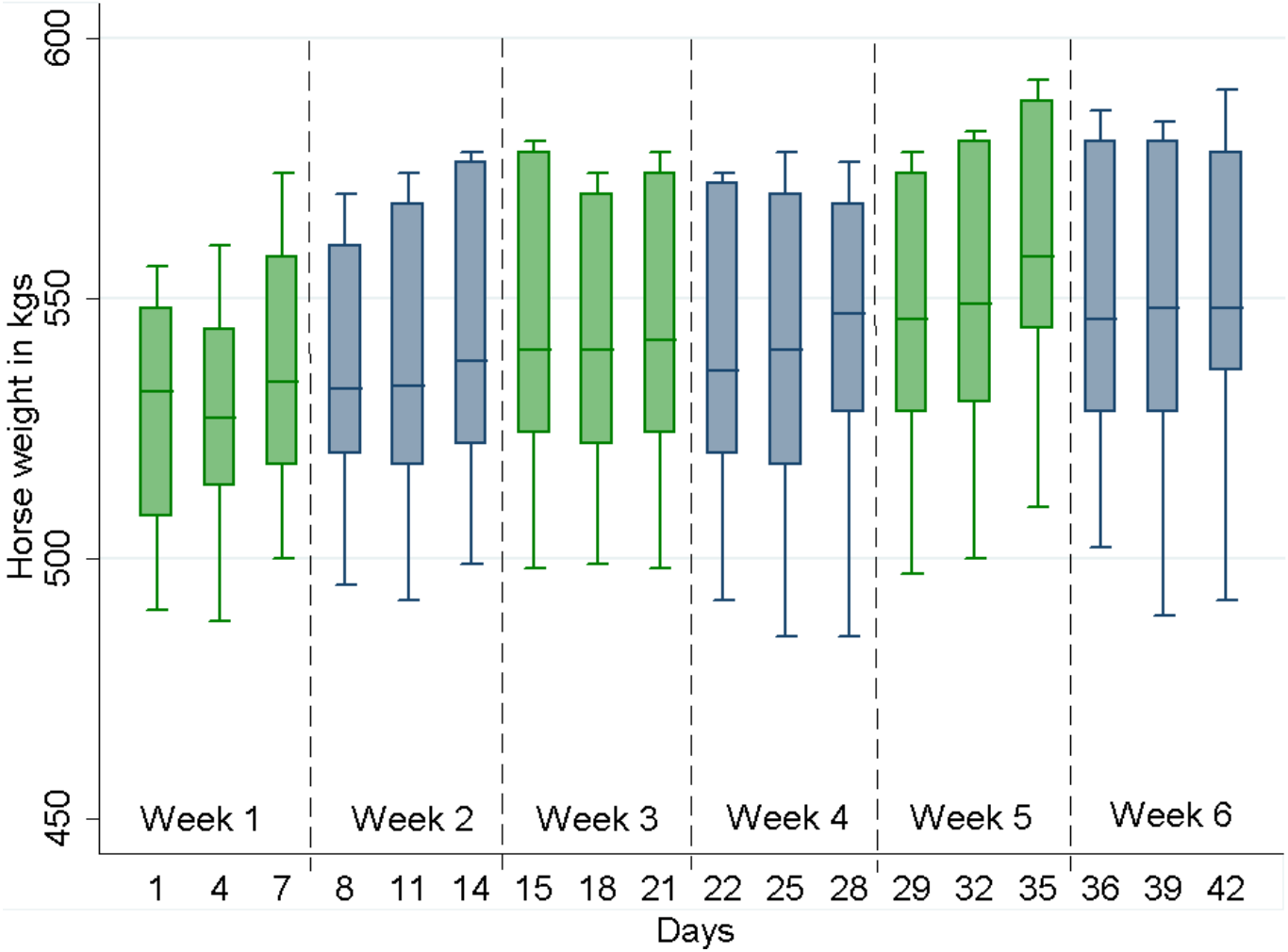
D) Retrieval of green markers. E) Retrieval of blue markers.

E-7. Example of behaviours described in the ethogram and observed during scan sampling of the six horses over the study period.

A) Eating B) Standing alert C) Resting D) Lying down E) Self grooming

F) Grooming on door G) Mutual grooming H) Oral behaviour I) Licking objects J) Crib biting

E-8. Body weight of the horses measured on Days 1, 4 and 7of each treatment block.



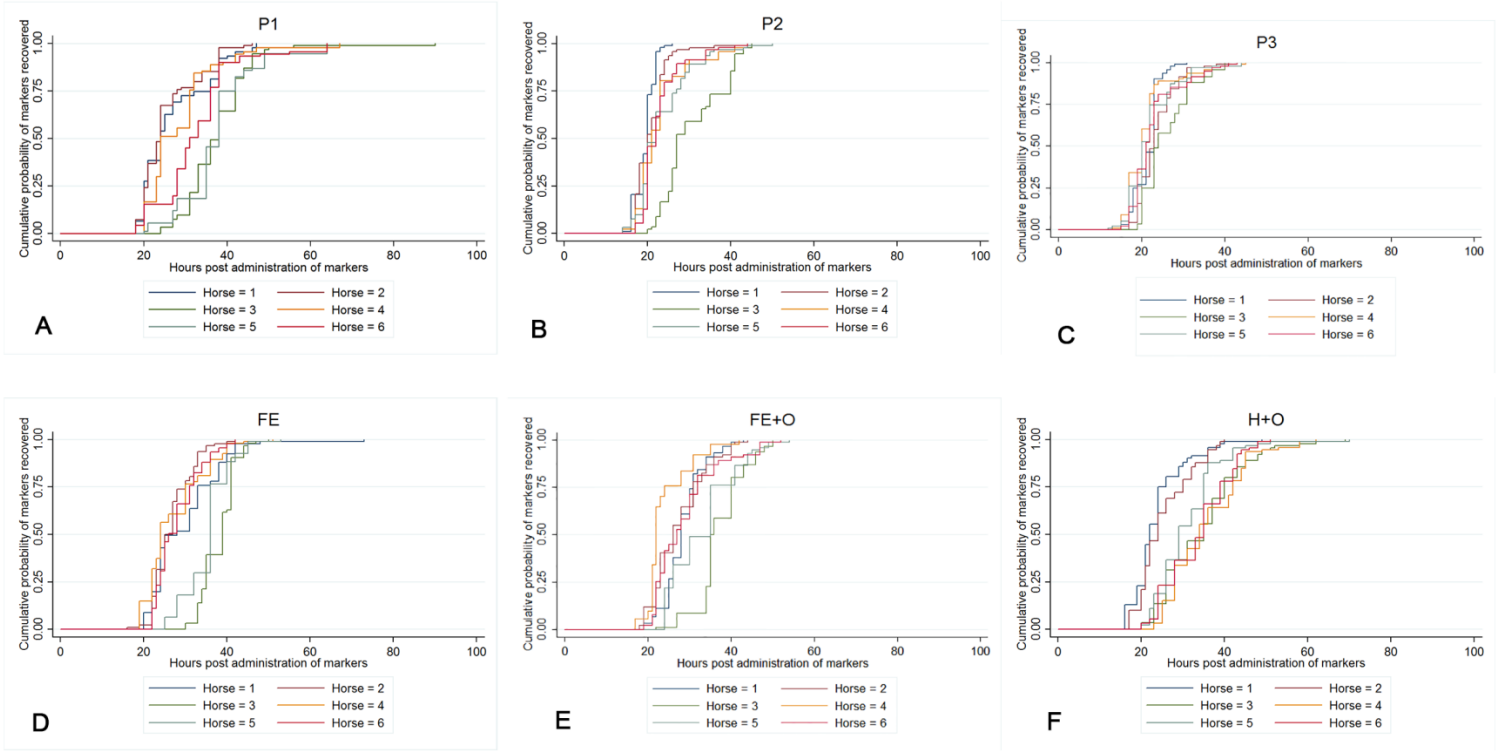
Legend: Green – period when horses were fed pasture (washout period). Blue – period when horses were fed the three treatment diets.

E-9. Time (h) for recovery of markers in the faeces of horses included in the study.

Diets	Horses	Time at recovery of markers (h)			
		T_1	**25%**	**50%**	**75%**
P1	H1	18	20	24	36
	H2	18	21	24	28
	H3	24	33	38	42
	H4	20	23	24	31
	H5	20	35	38	38
	H6	13	28	31	36
	Mean±SD	19±4	24±6	31±7	38±5
P2	H1	10	19	20	21
	H2	14	18	20	23
	H3	20	26	27	40
	H4	13	19	21	23
	H5	14	19	22	27
	H6	14	20	22	24
	Mean±SD	14±3	19±3	22±3	26±7
P3	H1	15	19	23	23
	H2	17	20	23	26
	H3	19	20	24	29
	H4	13	17	20	22
	H5	12	17	20	26
	H6	13	19	21	23
	Mean±SD	15±3	20±1	22±2	24±3
FE	H1	20	24	28	33
	H2	19	23	25	30
	H3	30	35	39	41
	H4	19	22	24	30
	H5	19	32	36	36
	H6	16	24	26	31
	Mean±SD	21±5	24±5	31±6	36±4
FE+O	H1	19	25	28	31
	H2	18	23	26	31
	H3	22	35	35	40
	H4	17	21	22	24
	H5	24	26	35	35
	H6	13	22	27	32
	Mean±SD	19±4	23±5	28±5	35±5
H+O	H1	16	21	22	24
	H2	17	21	24	30
	H3	16	26	35	40
	H4	13	28	34	42
	H5	17	26	29	35
	H6	20	28	35	39
	Mean±SD	17±2	24±3	29±6	36±7

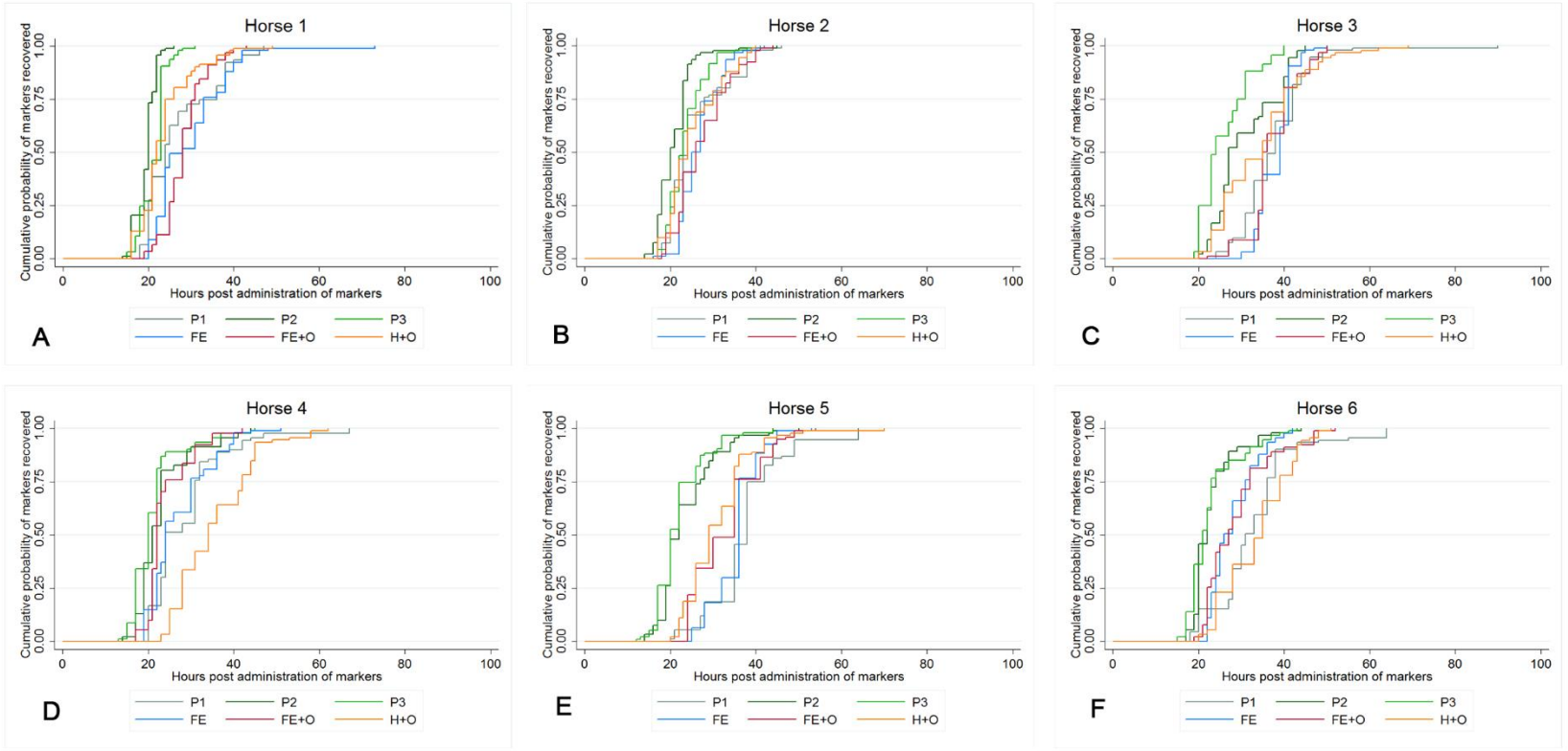
Legend: P1, P2 and P3 – cut pasture grass fed to the horses; FE – chopped ensiled forage; FE+O - chopped ensiled forage mixed with whole oats; H+O - ryegrass-clover hay fed with whole oats.

E-10. Additional file 1. Kaplan – Meier survival analysis of the cumulative percentage of markers recovered in the faeces of the six horses, on each diet fed during the six-week study period.



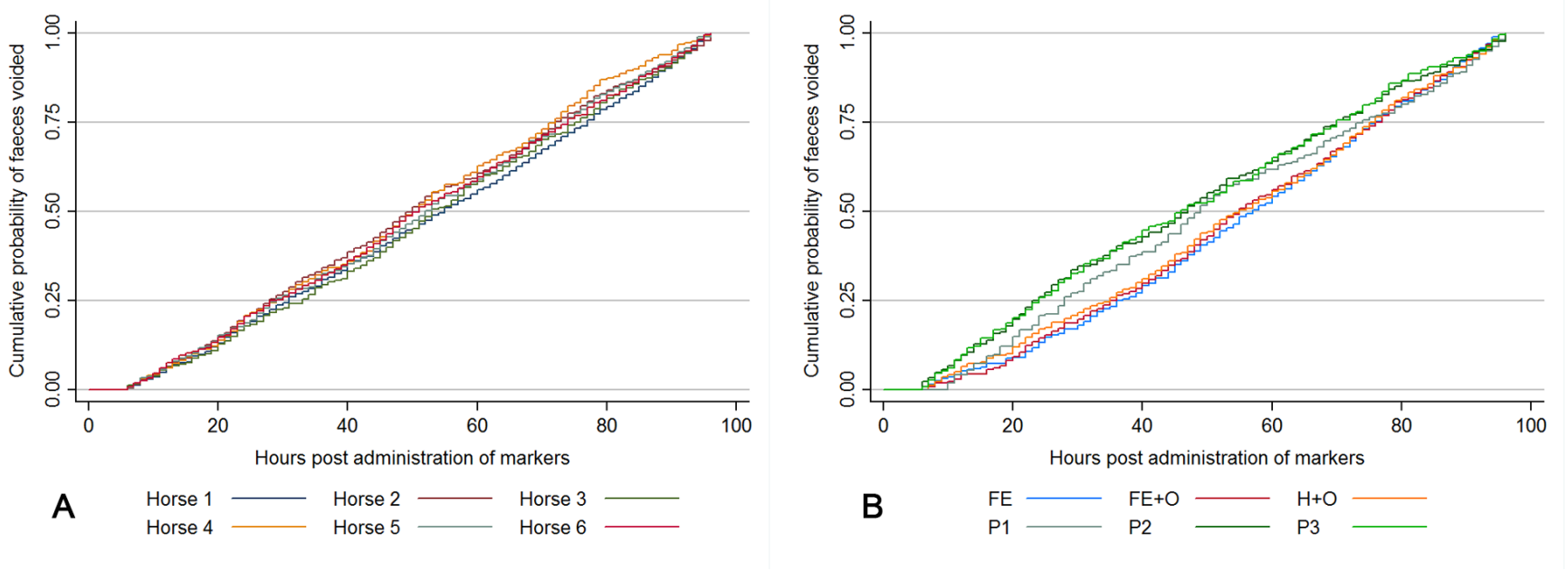
Legend: Within each diet, there was a significant difference in the marker recovery between horses (inter-horse variation) (log-rank test P<0.001 for all comparisons). P1, P2 and P3 – cut pasture grass fed to the horses in weeks 1, 3 and 5, respectively; FE – chopped ensiled forage; FE+O - chopped ensiled forage mixed with whole oats; H+O - ryegrass-clover hay fed with whole oats.

E-11. Additional file 2. Kaplan – Meier survival analysis of the cumulative percentage of markers recovered in the faeces of the six horses, stratified by horse across diets fed during the six-week study period.



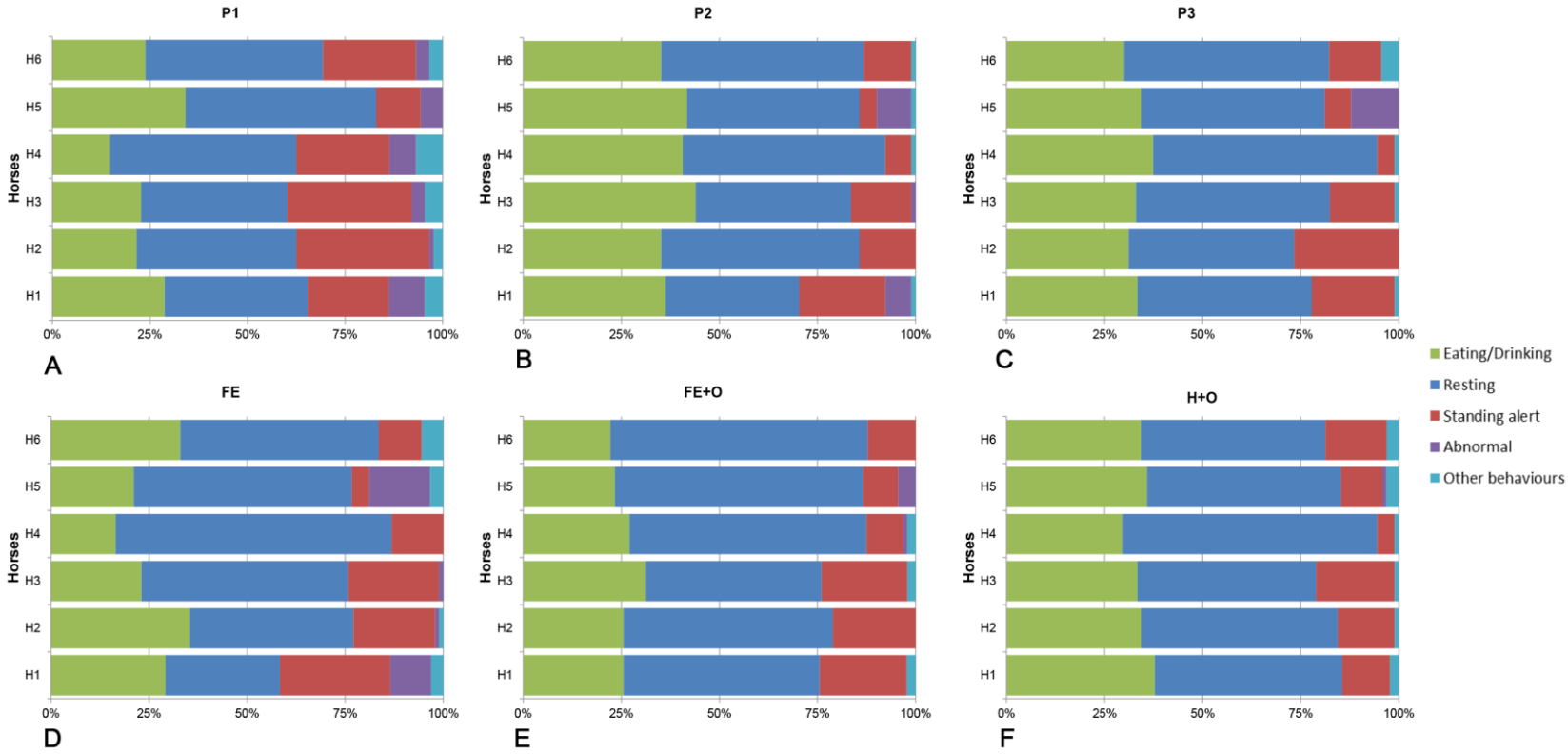
Legend: Within each horse (intra-horse variation), there was a significant difference in the marker recovery across diets (Log-rank test P<0.001 for all comparisons). P1, P2 and P3 – cut pasture grass fed to the horses in weeks 1, 3 and 5, respectively; FE – chopped ensiled forage; FE+O - chopped ensiled forage mixed with whole oats; H+O - ryegrass-clover hay fed with whole oats.

E-12. Additional file 3. Kaplan – Meier survival analysis of the cumulative frequency of faeces voided during the first four days of each dietary treatment block, stratified by horse and diet.



Legend: There was no effect of horse (Log-rank test, P=0.441) (Panel A), but diet had a significant effect on the survival functions of the cumulative frequency of faeces voided (Log-rank test, P=0.046) (Panel B). P1, P2 and P3 – cut pasture grass fed to the horses in weeks 1, 3 and 5, respectively; FE – chopped ensiled forage; FE+O - chopped ensiled forage mixed with whole oats; H+O - ryegrass-clover hay fed with whole oats.

E-13. Additional file 4. Behaviour of horses recorded over a 96-hour period, compared within diets fed during the study.



Legend: P1, P2 and P3 – cut pasture grass fed to the horses in weeks 1, 3 and 5, respectively; FE – chopped ensiled forage; FE+O - chopped ensiled forage mixed with whole oats; H+O - ryegrass-clover hay fed with whole oats. The 'abnormal behaviours' category included grooming, licking objects or premises, vocalising and non-repetitive oral or head movements and the 'other behaviours' category included repetitive box/ yard walking, crib biting and weaving.

CHAPTER 8

DYNAMICS OF FAECAL MICROBIOTA IN STABLED FORAGE-FED HORSES

(Feed and Microbiota Trial)

PRELUDE TO CHAPTER 8

Despite the expected individual variation between horses, the results of Chapters 5 and 6 showed that the faecal microbiota profile was diet-specific in a cohort of healthy horses fed forage-based diets. This diet-specific faecal bacterial community responded rapidly to an abrupt dietary change, with significant differences in the diversity and community structure observed in the first set of faecal samples collected on day 4 following dietary change (Chapter 5). However, the results of Chapter 7 showed that the quantity and type of feed consumed by the horses had an effect on the transit time of digesta, with a mean transit time of 23-24 h reported for horses consuming pasture *ad libitum*. This latter finding indicated that the change in diversity observed on day 4 in Chapter 5 may have occurred sooner. Fluctuations in the diversity of faecal bacteria in pasture-kept horses were also correlated with changes in pasture composition (Chapter 6). Therefore, it was of great interest to assess whether the diversity of the faecal bacterial community would stabilise in horses fed on a more consistent forage diet.

Chapter 8 is the second part of the feeding trial (described in Chapter 7), where the population dynamics of the faecal bacterial community of stabled horses was investigated following dietary transition between four different forage-based diets. The bacterial diversity was assessed using an advanced sequencing technique (Illumina MiSeq) and genomic DNA extracted from the faecal samples collected at specific time points during the study, to determine the rate of change in faecal bacterial diversity and its stability following dietary transition.

Supplementary information on the experimental design and some additional results are presented in the appendix for this chapter (Appendix F). Chapter 8 is based on a manuscript submitted to the Journal Public Library of Science: One (PLoS ONE).

Fernandes, K.A., Rogers, C.W., Gee, E.K., Kittelmann, S., Bolwell, C.F., Bermingham, E.N., Biggs, P.J. and Thomas, D.G. (Submitted on 10.06.2016). Resilience in the population dynamics of faecal microbiota in stabled Thoroughbred horses following abrupt dietary transition between freshly cut pasture and three forage-based diets. *PLoS ONE*.

CHAPTER 8: RESILIENCE IN THE POPULATION DYNAMICS OF FAECAL MICROBIOTA IN STABLED THOROUGHBRED HORSES FOLLOWING ABRUPT DIETARY TRANSITION BETWEEN FRESHLY CUT PASTURE AND THREE FORAGE-BASED DIETS

8.1 Abstract

The gastrointestinal microbiota of horses is diverse and sensitive to dietary manipulations, however the population dynamics following dietary transition is poorly understood. In the present study, the faecal microbiota of horses was investigated to determine how quickly the bacterial communities; 1) responded to dietary change, and 2) stabilised following abrupt dietary transition. Thoroughbred mares (n=6), were stabled for six weeks, intestinal markers were administered to measure digesta transit time, and then they were abruptly transitioned from consuming freshly cut pasture (weeks 1, 3 and 5), to conserved forage-based diets, both offered *ad libitum*. The conserved forage-based diets were fed according to a 3 × 3 Latin square design (weeks 2, 4 and 6), and comprised an ensiled chopped forage fed exclusively (Diet FE) or with whole oats (Diet FE+O), and perennial ryegrass hay fed with whole oats (Diet H+O). High throughput 16S rRNA gene sequencing was used to evaluate the diversity of the faecal microbiota. There were significant differences in alpha diversity across diets (Simpson's, Shannon-Wiener and Chao 1 indices, $P<0.001$ for each), and a significant effect of diet on the relative abundances of bacterial genera (ANOSIM, $P=0.001$), with clustering of samples observed by diet group. There were differences in the bacterial phyla across diets ($P<0.003$), with the highest relative abundances observed for Firmicutes (62-64%) in the two diets containing ensiled chopped forage, Bacteroidetes (32-38%) in the pasture diets, and Spirochaetes (17%) in the diet containing hay. Similar patterns of microbiota distribution were also observed at the genus level. Major changes in relative abundances of faecal bacteria corresponded with the cumulative percentage of intestinal markers retrieved in the faeces, with few significant differences between individual time points detected. A stable microbiota profile was observed in the treatment diets containing ensiled chopped forage after the 96 hours following dietary change. The present study confirmed that the diversity and community structure of the faecal bacteria in horses is diet-specific and

resilient following dietary transition, and emphasised the need to have modern horse feeding management that reflects the ecological niche, particularly by incorporating large proportions of forage into equine diets.

8.2 Introduction

As a hindgut fermenter, the horse is largely dependent on the production of volatile fatty acids (VFAs) for energy, with up to 60% of energy derived from the forage consumed by the horse and digested by microbial fermentation in the hindgut (Glinsky *et al.*, 1976, Janis, 1976). Typically, a horse's diet may comprise 50-100% forage, together with variable proportions of concentrate feeds including grain, grain-by-products and other supplementary feeds (Lewis *et al.*, 1995). Depending on individual horse requirements, preference or performance needs, horses may consume ~2% of their body weight (BW) of forage per day (on a dry matter (DM) basis), either in the form of fresh (pasture) or conserved (hay or haylage) forage (Anonymous, 2007).

In New Zealand, survey studies on the feeding management of leisure and competition horses showed that most horses and ponies are kept on pasture all year round (Fernandes *et al.*, 2014, 2015 and Verhaar *et al.*, 2014). Supplementary feeds are fed to meet additional energy requirements for performance, to overcome potential deficits with the seasonal availability of pasture, or to balance the diet (Fernandes *et al.*, 2014, and Verhaar *et al.*, 2014). Under this pasture-based management system, most horses are reported to be healthy and maintained BW and condition throughout the year (Fernandes *et al.*, 2014, 2015 and Fernandes *et al.*2016a).

The management of competition horses in New Zealand often involves rotations of short periods of stall confinement and concentrate feeding, followed by variable periods of time at pasture (Fernandes *et al.*, 2014 and Rogers *et al.*, 2007). Under these management systems, horses may undergo abrupt dietary changes, with the incorporation of grains or concentrate feeds to the diet to meet performance needs, or sudden changes in the type of forage fed in response to a lack of fresh or conserved forage (Williamson *et al.*, 2007). Abrupt changes in dietary management are a risk factor for gastrointestinal (GI) disturbances, potentially due to the negative effects observed on the population of GI microbiota (Cohen *et al.*, 1999, van den Berg *et al.*, 2013).

The function and stability of the GI microbiota is important for optimal health of the horse (Costa and Weese, 2012, Flint *et al.*, 2015). New sequencing technologies (using high-throughput next generation sequencing of the 16S and 18S rRNA gene amplicons) have advanced our understanding of the complexity, richness, and diversity of the equine GI and faecal microbiota, under various experimental conditions (Costa and Weese, 2012, Dougal *et al.*, 2013, Fernandes *et al.*, 2014). In healthy horses, the GI microbiota comprises a rich and diverse community, dominated by fermentative bacteria that are crucial for the efficient utilisation of nutrients from plant material (Moore and Dehority, 1993, Fernandes *et al.*, 2014). However, significant changes in the diversity of GI microbiota have been reported following dietary manipulations (including the feeding of high-starch diets, different starch sources, or more highly digestible diets) (Daly *et al.*, 2012, Kristoffersen, 2014, Destrez *et al.*, 2015, Hansen *et al.*, 2015, Harlow *et al.*, 2016), and GI disorders (including fermentative acidosis, colitis, carbohydrate-induced laminitis, gastric ulcers and equine metabolic syndrome) (de Fombelle *et al.*, 2001, Milinovich *et al.*, 2006, Al Jassim and Andrews, 2009, Costa *et al.*, 2012, Dong *et al.*, 2016, Elzinga *et al.*, 2016). Transportation stress, administration of anaesthetics and antibiotics, and changes in management can also disrupt the population of GI microbiota, and these disruptions may be sustained for prolonged periods (weeks to months) (Harlow *et al.*, 2013, Costa *et al.*, 2015c, Schoster *et al.*, 2015).

Previous studies in New Zealand showed that the faecal bacterial community in horses was diet-specific, and the diversity and community structure changed within four days following an abrupt dietary transition from an ensiled chopped forage diet (fed in stables) to grazing on pasture (Fernandes *et al.*, 2014). In healthy horses grazing on pasture, the diversity and community structure of the faecal bacteria also fluctuated with seasonal changes in the pasture composition (Fernandes *et al.*, 2016a), indicating that changes in dietary substrate affect the microbiota populations in the hindgut. A subsequent study showed that the transit time of forage diets through the GI tract varied between diets depending on the moisture content of the forage, and the feeding behaviour and the dry matter intake of horses consuming the diets (Fernandes *et al.*, 2016b). It was hypothesised that the rate of change in the diversity of faecal microbiota would be associated with the transit time required for passage of digesta through the GI tract. Previously, changes in microbial activity were observed as early as 5 hours in the caecum and 29 hours in the colon following a dietary change in the quantity of grain fed to horses (de Fombelle *et al.*, 2001). Therefore, the changes in faecal microbiota

observed at four days (96 hours) following abrupt dietary transition from one forage to another in our previous study (Fernandes *et al.*, 2014), may have occurred sooner.

The objectives of the present study were therefore to; 1) determine whether the faecal microbiota of horses was diet-specific even when forage comprised the major proportion of the diet, 2) determine if the microbial populations changed before 96 hours, and quantify the rate of change following abrupt dietary transition from pasture to three conserved forage-based diets, and 3) determine if the microbiota profile stabilised when the diets were fed over a short term. The outcomes of this study will facilitate our understanding of the dynamics and resilience of the faecal microbiota populations in response to abrupt dietary change.

8.3 Materials and Methods

8.3.1 Ethics statement

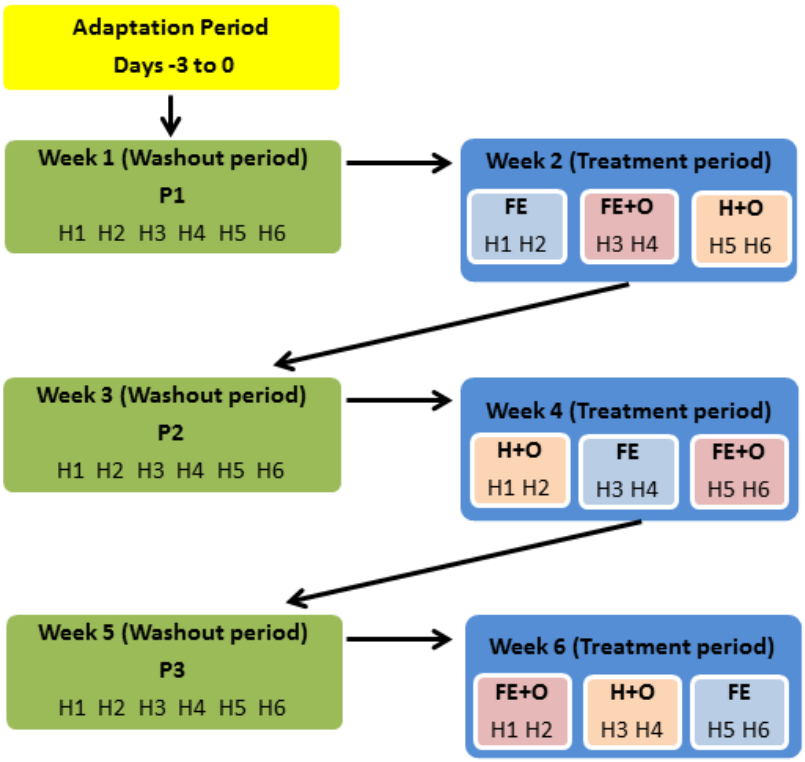
The use of animals, experimental procedures and collection of the faecal samples for the study, complied with the code of ethical conduct for the use of live animals for research, testing and teaching, and were approved by the Massey University Animal Ethics Committee (MUAEC Protocol number 14/35), Massey University, Palmerston North, New Zealand. An on-call veterinarian examined the horses on a weekly basis to ensure that all horses remained clinically normal during the study period.

8.3.2 Experimental design and trial management

The study was conducted from 14th July to 24th August 2014 (42 days in winter), as part of an investigation on the transit time of digesta in forage-fed horses. Detailed information on the experimental design and study protocol has been described previously (Fernandes *et al.*, 2016b). Briefly, six Thoroughbred mares of similar age, BW and body condition score were enrolled in the six-week study that was sub-divided into six dietary treatment blocks, each of seven days duration (Figure 8.1). The horses had been previously maintained on pasture (Diet PP) as part of a herd on a commercial equine breeding farm. In the six months preceding the study, all animals were reported to have been in good health, and had not received any antibiotic treatments. Three days prior to the beginning of the study, the horses were transported a short distance (~20 km) to the trial site and transferred to individual paddocks to facilitate adaptation to the new environment. A faecal sample was collected from

each horse within two hours of arrival, by using a forceps to select an uncontaminated portion from the centre of a freshly voided faecal mass. A sub-sample was examined for faecal egg count of GI parasites and the remaining portion was transferred into 2 ml polyethylene cryogenic vials (Ray Lab Ltd., Auckland, New Zealand) and snap frozen in liquid nitrogen and stored at -80°C (day 0 faecal sample).

Figure 8.1. Illustration of the experimental design for the six-week study period.



Legend: The horses (n=6) were on an adaptation period for three days prior to beginning the six-week study. At the start of each dietary block, the horses were administered with intestinal markers (green: washout period and blue: treatment period) via a nasogastric tube. The horses were abruptly transitioned to feeding on cut pasture during the washout period in weeks 1, 3 and 5 (Diets P1, P2 and P3). Three conserved forage based-diets were fed according to a 3 × 3 Latin square design during the treatment period in weeks 2, 4 and 6. The treatment diets were FE (chopped ensiled forage), FE+O (chopped ensiled forage fed with whole oats), and H+O (hay fed with whole oats).

During the six-week trial period, the horses were individually stabled in 3 × 3.5 m loose-boxes with sawdust bedding, turned-out into individual yards for 30 min twice daily,

and were provided with *ad libitum* access to water, a trace-mineral salt block and feed. The horses were offered four types of forage-based diets over the six-week period. During weeks 1, 3 and 5 of the trial (washout periods), the horses were fed freshly cut grass obtained from perennial ryegrass-clover pasture (Diets P1, P2 and P3). This was provided in two hay nets and one bucket that were refilled as per individual horse requirements (when empty or at ~ 4 hour intervals) to provide at least 2.5-3.0% BW of feed (DM basis). The quantity of cut pasture offered during the initial diet period P1 was restricted compared to *ad libitum* access to feed in diet periods P2 and P3.

Using a 3 × 3 replicated Latin square design, three pairs of horses that were fed freshly cut pasture (washout period) were abruptly transitioned onto three randomly-allocated conserved forage-based diets during weeks 2, 4 and 6 (Figure 8.1). The three treatment diets were: a commercial ensiled chopped forage (Diet FE; FiberEzy® containing timothy grass, Fiber Fresh Feeds Ltd., Reporoa, New Zealand), a commercial ensiled chopped forage mixed with whole oats (Diet FE+O), and a perennial ryegrass hay fed with whole oats (Diet H+O). The ensiled chopped forage component of the diet used during the trial was prepared as one batch, processed under similar conditions on the same day, and stored in double plastic-wrapped packaging for ~6 months prior to the study. The hay was harvested and processed as one batch, and was stored in a dry covered shed for ~6 months prior to the study. Diet FE was offered at a minimum of ~2.5-3.0% BW (DM basis) as two feeds at 0800 and 2000 hours. Diet FE+O and H+O were also provided twice daily at the same times, and to ensure *ad libitum* feeding at ~2% BW (DM basis)/horse/day, additional quantities of feed were provided when less than 25% of the feed was remaining. The quantity of oats was calculated based on 50% of the minimum daily energy requirements for maintenance (DER_m) for a 500 kg horse (~35 MJ/horse/day) (Anonymous, 2007), which was equivalent to 2.5 kg DM/horse/day, divided into the two feedings. Refusals were collected twice daily and weighed to determine the amount of feed consumed. Additional details on the nutrient composition of the dietary components and feeding management are described previously (Fernandes *et al.*, 2016b).

8.3.3 Data recording and sample collection

The general health, appetite and behaviour of the horses were assessed daily by one of the study personnel (KAF). Body condition scores (BCS, 1-9 (Henneke *et al.*, 1983)) and BW of the horses were recorded on the 1st, 4th and 7th day of each week, to ensure that the horses maintained BW and condition during the study. Representative feed samples of each

diet were collected and analysed to determine the nutrient content using a combination of near infrared and plasma spectrophotometer techniques (Equi-Tech, Equi-Analytical Laboratories, Ithaca, NY) as described previously (Fernandes *et al.*, 2016b)).

During the trial period, all faecal matter produced by the horses was collected from the bedding in the stables at hourly intervals on days 1-4 (starting at 6 hours after nasogastric administration of the markers on day 1 and continuing until 96 hours post-administration of markers on day 4). A single representative sample of faecal matter was collected on days 5, 6 and 7 of each treatment block. Individual sub-samples were transferred into 2 ml polyethylene cryogenic vials (Ray Lab Ltd., Auckland, New Zealand), and immediately snap frozen in liquid nitrogen (Appendix F-1). The samples were transferred to a -80°C freezer within two hours of collection and stored until laboratory analysis of bacterial diversity using next generation sequencing technology.

A total of 3,348 faecal samples (93 samples × 6 horses × 6 weeks) were collected during the study period. A sub-set of 377 samples were selected for further analysis, which consisted of those collected at specific time points (0, 12, 24, 48, 72, 96, 120, 144 and 168 hours) after dietary change and those containing the first marker recovered (M1), and cumulative percentages of markers recovered in the faeces (M25, M50 and M75) (Appendix F-2, Table S1; (Fernandes *et al.*, 2016b)).

8.3.4 DNA extraction, amplicon library construction and sequencing

As described previously (Fernandes *et al.*, 2016a), nucleic acids were extracted from 100 mg of each faecal sample (n=377) using a combined bead-beating, phenol-chloroform and column purification protocol and QIAquick 96 PCR purification kit (Qiagen, Hilden, Germany), and eluted in 80 µl elution buffer. After quantification and quality assessment, all (n=377) gDNA samples were normalised to 5 ng/µl gDNA per sample and bacterial 16S rRNA gene libraries were constructed using the Illumina single-step PCR library preparation method (Illumina, San Diago, CA, USA, www.illumina.com). Seven blank samples containing water, were included as internal controls across the four plates (total 96 × 4 plates; n=384 samples). Further details on the PCR protocol and the primer pair used to target the V3-V4 hypervariable region of the 16S rRNA gene are described previously (Fernandes *et al.*, 2016a), and the unique 8 bp dual-index barcode sequences (Nextera DNA library preparation kit, Illumina) used for individual sample identification are given in Appendix F-2, Table S1. Following validation of the purified sequence libraries using a DNA 1000 labchip on a 2100 Bioanalyzer

(Agilent Technologies, Santa Clara, CA, USA), the 16S amplicon libraries (n=384) were pooled in equimolar concentrations into two pools with 192 libraries each. The pooled libraries were denatured using fresh NaOH, and spiked with 10% volume of a PhiX control library (PhiX control kit v3, Illumina), before loading onto 2 × 300 base paired-end sequencing runs (192 libraries per run) using the Illumina MiSeq platform (MiSeq 600 cycle kit, version 3 chemistry, Illumina) (Kozich *et al.*, 2013, Fadrosh *et al.*, 2014).

8.3.5 Bioinformatics and statistical analyses

Quality control analysis was performed on the original sequences using three processes: SolexaQA++, FastQC and FastQscreen, which are described in detail previously (Cox *et al.*, 2010, Fernandes *et al.*, 2016a). Briefly, the raw sequence reads obtained from the Illumina MiSeq runs were aligned against the PhiX genome, and the PhiX sequences detected were removed, leaving the unaligned sequences that were included in further downstream analysis. The SAM and fastq files were reconstructed, Illumina adaptors and PCR primers were removed, and the sequence reads were assigned to corresponding samples by examining the 8 bp barcode sequence (Kozich *et al.*, 2013). Since the reverse sequences (read 2) generated on the MiSeq runs were of poor quality, these sequences were discarded, and only the forward sequences (read 1) were used for further downstream analysis. The processed sequences were trimmed to their longest contiguous segment for which error probabilities were greater than a threshold of 0.003 (equivalent to quality of ~25 Phred score) using the DynamicTrim application, and short reads (<250 bp) were removed from the bacterial sequence library using the LengthShort application (Cox *et al.*, 2010). All sequences that did not meet the above quality filtering criteria were excluded from further downstream analysis. The project is registered with NCBI PRJNA326194, and the sequence data generated in this study are available via the Sequence Read Archive under the accession number SRP076876.

Ecological analysis on the retained sequence data was performed using the QIIME package (Quantitative Insights Into Microbial Ecology, v1.8) (Caporaso *et al.*, 2010b), as per the protocol described previously (Fernandes *et al.*, 2016a). The sequences obtained from the two MiSeq runs were checked for run-to-run variation by comparing the beta diversity clustering of samples that were expected to be similar (i.e. faecal samples from diet groups P2 and P3 that were sequenced on different runs), using the unifrac analysis in QIIME. The sample with the lowest number of sequences was identified, and all samples were normalised to this minimum value, so that all samples could be included in the analysis across diet groups

and time points. Bacterial species richness in the samples were assessed on the Collector's curves, and the alpha diversity rarefaction analysis was computed for a maximum of 3,022 sequences per sample, which was visualised by diet and horse parameters included in the metadata (Appendix F-2, Table S1). Subsequently, the original OTU table was rarefied to a subsample of 3,022 sequences per sample, and Good's coverage (mean percentage ± standard deviation [SD]) was estimated to ensure representative subsampling (Good, 1953, Gihring *et al.*, 2012). Alpha diversity was evaluated at the OTU level using the PAST software (version 3.08, *http://folk.uio.no/ohammer/past/*), and included the Simpson's index of diversity, Shannon-Wiener index of entropy and the Chao1 index for species richness (Simpson, 1949, Hammer *et al.*, 2001, Spellerberg and Fedor, 2003, Gotelli and Colwell, 2011). Relative abundance of bacterial taxa were summarised at phylum and genus levels. Bacterial phyla and genera with relative abundances <1% in all samples were grouped as "other phyla" and "other genera", respectively. Beta diversity was evaluated on a genus level using the QIIME pipeline (Caporaso *et al.*, 2010b), using bacterial taxa that represented ≥1% of the total community, in at least one sample, and differences between bacterial communities were determined using the Bray-Curtis dissimilarity metric. Clustering of samples was visualised by principal coordinate analysis (PCoA) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using various software tools (Tamura *et al.*, 2013, Vazquez-Baeza *et al.*, 2013), as described previously (Fernandes *et al.*, 2016a).

The data generated were imported into a spreadsheet (Microsoft Excel, version 2010, Microsoft Corp., Redmond, WA), re-formatted where necessary, and explored for inconsistencies or outliers, before tests for statistical significance were conducted. Statistical analysis was performed using the SAS v9.4 (SAS Institute Inc., Cary, NC, USA) and STATA v12.1 (Stata Corp., College Station, TX, USA) software packages. All variables were checked for normal distributions using the Shapiro-Wilk test (significance value of $P \leq 0.05$). Data are presented as mean ± SD for parametric data or median ± IQR for non-parametric data. Time points for markers are labelled as M1 representing the time when the first marker was recovered in the faeces, and M25, M50 and M75 representing the time when 25%, 50% and 75% of markers were recovered in the faeces. Time points in hours are labelled as T0-168 representing the hours following dietary change. All statistical tests to determine significant differences across diet groups were performed using data obtained from samples collected on days 5, 6 and 7 of each treatment block (T120, T144 and T168). Alpha diversity indices were calculated using the PAST software (version 3.08, (Hammer *et al.*, 2001)). Significant

differences in alpha diversity indices were determined between diet groups (P1, P2, P3, FE, FE+O and H+O) using the Kruskal-Wallis test, and between time points (T120, T144 and T168) using the Friedman's test. A significance value was set at $P \leq 0.05$ with a Steel-Dwass test or Bonferroni adjustment for multiple comparisons, as required (SAS v9.4 and PAST v3.08).

The effect of diet and time point on the beta diversity of bacterial taxa was tested using the analysis of similarity (ANOSIM) option with the QIIME script *compare_categories.py* (Caporaso *et al.*, 2010b). Results were considered significant at $P<0.05$ and values between 0.05 and 0.10 were considered to reflect trends. The mean relative abundances for four dominant phyla and six dominant genera across the diet groups are presented using stacked bar charts, and data on the remaining taxa are provided in the supplementary material. Significant differences in the relative abundance of bacterial taxa between diet groups (P1, P2, P3, FE, FE+O and H+O) were determined by the *group_significance.py* script in QIIME using the Kruskal-Wallis test with Bonferroni adjustment for multiple comparisons (adjusted P values were $P<0.003$ for phylum level and $P<0.001$ at genus level comparisons). Significant differences in the relative abundance of bacterial taxa between time points (T0, T24, T48, T72 and T96; T0, M1, M25, M50 and M75; and T120, T144 and T168) were determined using the Friedman's Test with Bonferroni adjustment for multiple comparisons. Post-hoc analysis to determine significant differences between individual time points (T0 vs. M1 and T0 vs. T24) was conducted for the most dominant phyla using the Wilcoxon signed-rank test with Bonferroni adjustment for multiple comparisons. Data obtained from all samples were included when examining the dynamics and stability of the bacterial communities, and are presented as line graphs of the relative abundances of the most dominant taxa (mean + SD), the difference between the mean relative abundance at consecutive time points (calculated as x-y/x for Firmicutes and Bacteriodetes), and the Firmicutes:Bacteroidetes (F:B) ratio. Comparisons between transit time of digesta and the relative abundance of taxa at the phylum and genus levels, within individual horses and diets, are presented using a matrix of bar charts.

8.4 Results

8.4.1 Animal health monitoring

All horses maintained BW and condition, and showed no signs of health problems throughout the study period (Fernandes *et al.*, 2016b).

87.4.2 Population dynamics of the faecal bacterial community

8.4.2.1 Metrics of sequencing data and rarefaction analysis

The two runs on the Illumina MiSeq platform generated ~181 million sequences. There was no significant run-to-run variation between the samples from diet groups P2 and P3. The mean number of sequences per sample and the metrics of data for sequences that passed quality filtering are shown in Table 8.1. The reverse sequences (read 2) obtained from the MiSeq runs were of poor quality, and hence were discarded. This lowered the length of sequences per sample (mean length 283 bp). After normalisation at 3,022 sequences per sample, 9,008 OTUs were detected at 97% similarity across the samples (n=377). The mean number of OTUs per sample was 713 (SD 93, range 347 – 916). A total of 1,139,294 bacterial sequences were included in the downstream analysis, wherein at least 26 phyla comprising of at least 408 genera were detected.

Table 8.1. Metrics of sequencing and quality screening.

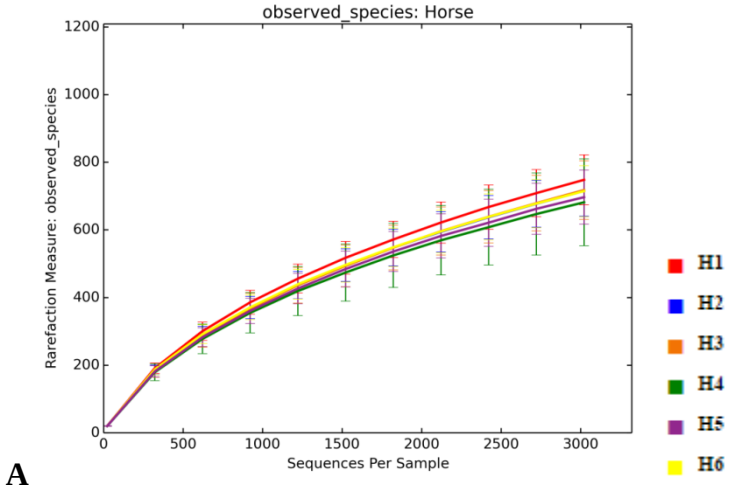
Details	Bacterial sequences
Initial processed reads §	
Total number of reads	181,673,688
Mean number of reads per sample	345,288
(range)	(87,312 – 748,408)
High quality reads used in downstream analysis †	
Total number of reads	5,531,552
Mean number of reads per sample	14,673
(range)	(3,022 – 43,275)
Mean length of reads (bp)	283
(range)	(250 – 301)
Total OTUs detected at 97% similarity	14,889

§ After using SolexaQA++, fastaQC, fastQscreen, BWA PhiX, fastq-mcf
† After using DynamicTrim, LengthShort, Chimera check

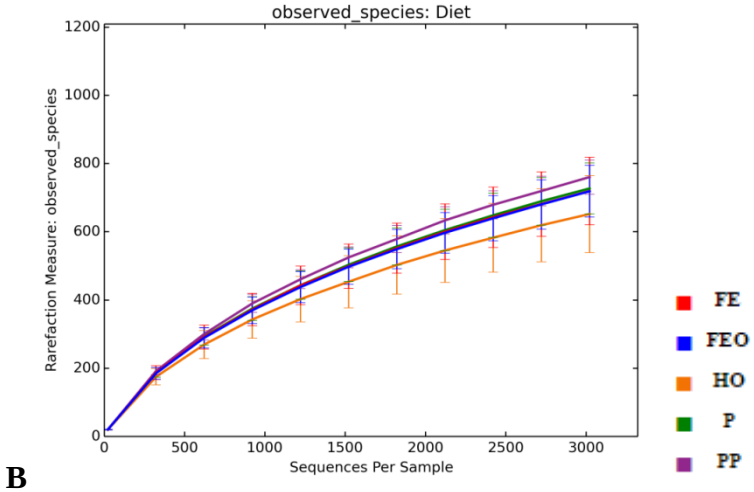
The rarefaction curves generated using the observed species metric for alpha diversity of the bacterial sequences are displayed in Figure 8.2. Novel OTUs were identified at the end of sub-sampling at 3,022 sequences per sample, which may indicate a lack of complete sampling effort. However, the rate of new OTU discovery was relatively limited around that sub-sampling threshold. Good's coverage estimates on the normalised OTU table indicated that the sampling depth had adequately captured a large part of the OTU diversity in all

samples, with the mean coverage being 88 ± 2%. The proximity of the rarefaction curves indicated small variation in the mean number of OTUs observed between the six horses enrolled in the study (682–748 OTUs; difference 66 OTUs; Figure 8.2A). Greater variation was observed in the mean number of OTUs detected across the diet groups (652–761 OTUs; difference of 109 OTUs; Figure 8.2B). Horses grazing in a paddock (Diet PP; day 0 samples) had a higher mean number of OTUs detected (761 ± 50 OTUs), than when confined in stables and fed forage-based diets during the study (Diets P1, P2 and P3). The mean number of OTUs detected was similar when the horses were fed cut pasture (Diet P; 727 ± 75 OTUs) and the two ensiled chopped forage diets (Diets FE; 719 ± 99 and FE+O; 719 ± 76), and lowest when the horses were fed hay (Diet H+O; 652 ± 113), indicating some differences in the bacterial diversity across diets. There were also some differences in the number of observed species across time points (Figure 8.2C).

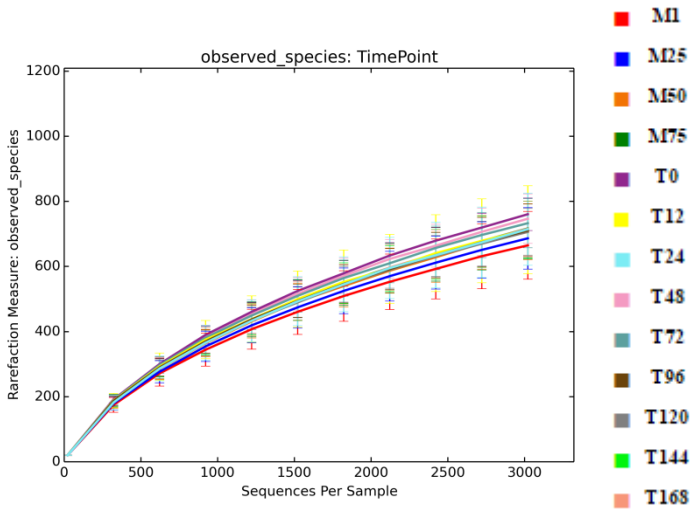
Figure 8.2. Rarefaction curves of the number of observed species presented by horse, diet and time points.



A



B



C

Legend: Panel A shows the rarefaction curves for the six horses (labelled H1-H6). Panel B shows the rarefaction curves presented by diets. The diets were labelled as PP (grazing on pasture in paddocks on day 0), P (cut pasture fed in stables, representing diet periods P1, P2 and P3 together), FE (ensiled chopped forage fed in stables), FE+O (ensiled chopped forage fed with whole oats in stables), and H+O (hay fed with whole oats in stables). Panel C shows the rarefaction curves for the time points, labelled as M1 (recovery of 1st marker in the faeces), M25, M50 and M75 (represent timepoints when 25%, 50% and 75% of markers were recoverd in the faeces), T0 (day 0 samples at the beginning of the trail), and T12-168 (represent the timepoints in hours following dietary transition).

8.4.2.2 Effects of diet on the diversity of the faecal bacterial community

The diets investigated showed high diversity of bacterial genera, with mean Simpson's diversity indices ≥0.89 for all diets. There were significant differences in the alpha diversity indices of bacterial genera across diets (P<0.001; Table 8.2).

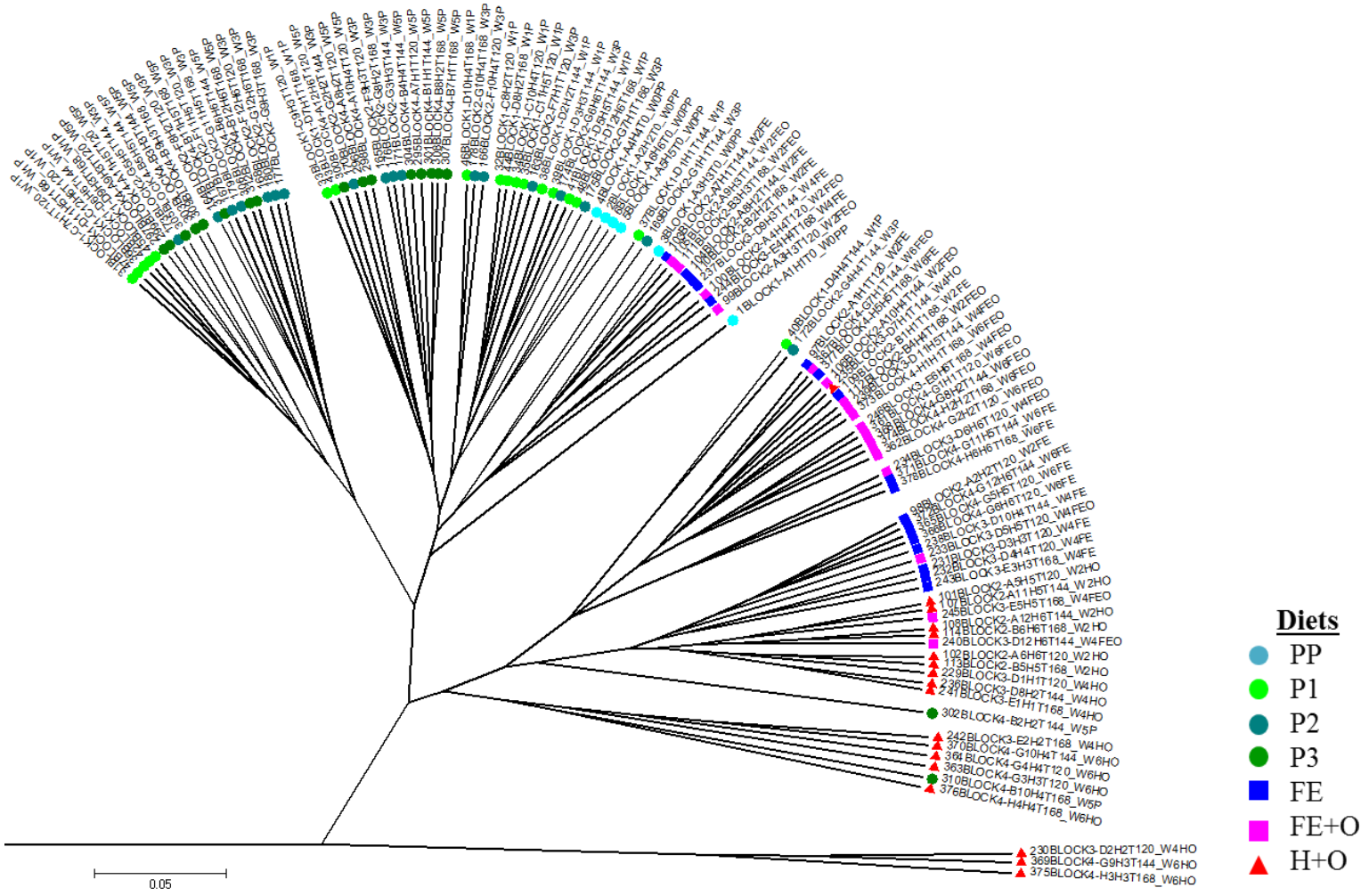
There were no significant differences in the alpha and beta diversity between samples from time points T120, T144 and T168 (i.e. faecal samples collected on days 5, 6 and 7, respectively) at the genus level (ANOSIM, P=0.754; Appendix F-3, Table S2). However, there were significant effects of diet on the beta diversity of the bacterial genera in the faeces (ANOSIM, P=0.001). Principal coordinate analysis at genus level showed clustering of samples by diet, with ~48% of the variation explained on three principal coordinate axes (Appendix F-4, Figure S1). The UPGMA dendrograms showed that the faecal bacterial community of horses when fed Diet H+O clustered separately to the Diets P1, P2, P3, FE and FE+O (Figure 8.3). The diets containing ensiled chopped forage (FE and FE+O) appeared as one cluster on the PCoA plots (Appendix F-4, Figure S1). While the diets containing cut pasture were another cluster, Diet P1 (restricted cut pasture) appeared to cluster separately from Diets P2 and P3 (*ad libitum* cut pasture). Although there was some overlap, Diet P1 and Diet PP clustered more closely and were positioned between the Diets P2, P3 and Diets FE, FE+O on the PCoA plots and UPGMA dendrograms (Figure 8.3 and Appendix F-4, Figure S1).

Table 8.2. Comparison of the median alpha diversity of the bacterial genera across diets.

Alpha diversity index	Diets						P value*
	P1	P2	P3	FE	FE+O	H+O	
Simpson's (diversity)	0.89	0.89	0.91	0.90	0.89	0.89	<0.001*
(IQR)	(0.88-0.90)	(0.88-0.90)	(0.90-0.92)	(0.89-0.91)	(0.88-0.90)	(0.88-0.90)	
Shannon-Wiener (entropy)	2.74	2.75	2.91	2.78	2.80	2.74	<0.001*
(IQR)	(2.67-2.80)	(2.70-2.80)	(2.87-2.98)	(2.77-2.91)	(2.68-2.88)	(2.64-2.80)	
Chao 1 (richness)	61	65	69	62	60	62	<0.001*
(IQR)	(59-65)	(63-70)	(65-72)	(56-65)	(56-63)	(58-65)	

Legend: *Kruskal-Wallis test; level of significance was P≤0.05 with Steel Dwass test for multiple comparisons. The values for alpha diversity indices are presented as median (IQR - interquartile range). Samples collected on days 5, 6 and 7 of each treatment block were included in the analysis (see methods). Diets are represented as P1, P2, P3 - cut pasture fed during weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage fed with whole oats; and H+O - hay fed with whole oats.

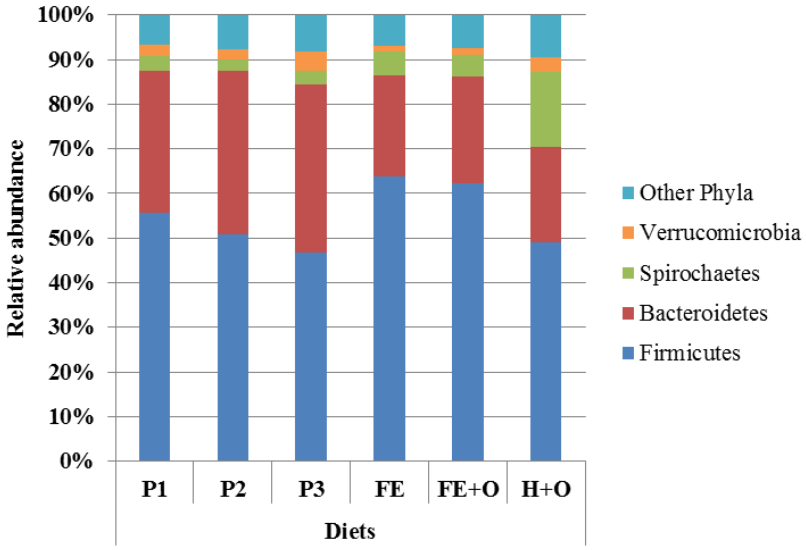
Figure 8.3. UPGMA dendrogram of bacterial communities (genus-level) in the faecal samples of horses fed pasture (P1, P2, P3) and three conserved forage diets (FE, FE+O, H+O) on days 5, 6 and 7 of the study period.



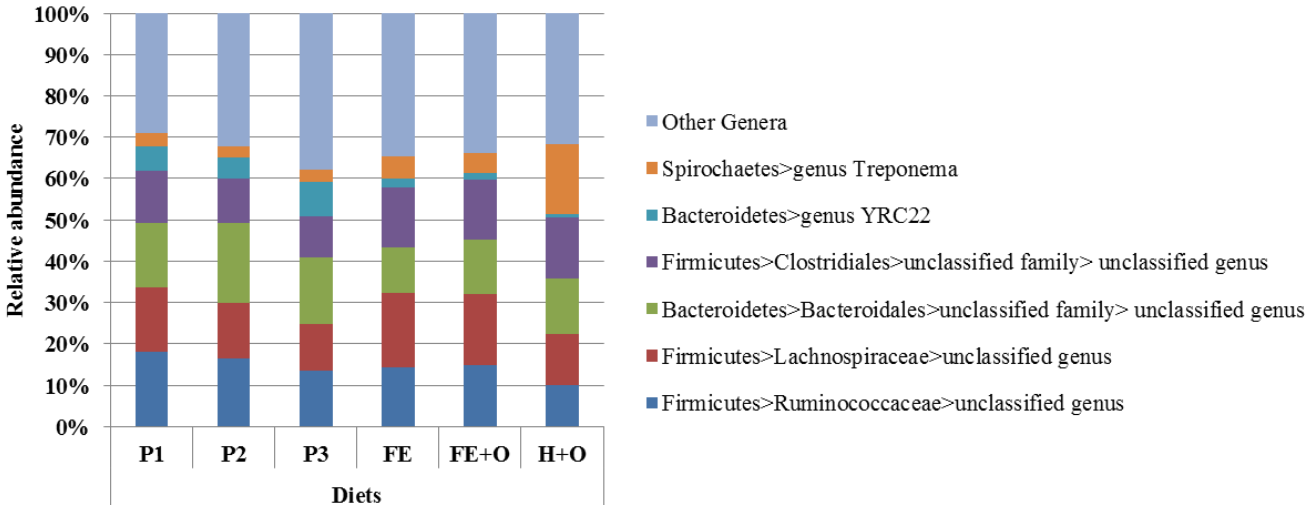
Legend: Unweighted pair group method with arithmetic mean (UPGMA) generated using the Bray Curtis dissimilarity metric of bacterial genera in all faecal samples included in the study. Samples collected on days 5, 6 and 7 of each treatment block were included in the analysis (see methods). Samples are colour-coded to visualise clustering by diet. Each sample is labelled using the prototype: Sample-Block-Well-Horse-Timepoint-Week-Diet (e.g. 31BLOCK1-C7H1T120_W1P reads as sample 31, on block 1, well C7, from horse 1, at time point 120 h, in week 1, on pasture diet), with blocks 1-4, wells A-H; horse numbers H1-6; time points T144-168; weeks 1-6. Diets are represented as PP (teal circles; grazing pasture in paddock), P1,2,3 (light or dark green circles; cut pasture fed in stable), FE (Blue square; ensiled chopped forage fed in stable), FE+O (Pink square; ensiled chopped forage fed with whole oats in stable), H+O (red triangle; hay fed with whole oats in stable). The scale bar indicates 0.05 dissimilarity between samples.

The clustering in beta diversity observed in Figure 8.3 and Appendix F-4, Figure S1 was due to significant differences in the relative abundances of bacterial phyla across the diets (Appendix F-5, Table S3). Despite the differences in relative abundances, the faecal bacterial community across all diets was dominated by the same two phyla, the Firmicutes and the Bacteroidetes, which comprised between 70-90% of the bacterial community, except in the diet containing hay, where Spirochaetes were over represented (Figure 8.4A). Diets FE and FE+O had the highest relative abundance of Firmicutes (62-64%), and lowest relative abundance of Verrucomicrobia (~1%), when compared to the other diets. The relative abundance of Bacteroidetes (32-38%) was highest in Diets P1, P2 and P3 (fresh cut pasture), whereas all three conserved forage-based diets (FE, FE+O and H+O) had lower relative abundance of Bacteroidetes (21-24%). Diet H+O had the highest relative abundance of Spirochaetes (17%), when compared to all other diets (Figure 8.4A, Appendix F-5, Table S3). These differences in community structure were reflected in similar patterns of distribution in relative abundances at the genus level (Figure 8.4B). The genus *Treponema* (Spirochaetes) was highest in Diet H+O, and unclassified genera within the order Clostridiales and family Lachnospiraceae (both Firmicutes) were highest in the Diets FE and FE+O, and the genus YRC22 and an unclassified genus within the order Bacteroidales (both Bacteroidetes) were highest in Diets P1, P2 and P3. P-values for the comparison of relative abundances of the bacterial genera across diets are shown in Appendix F-6, Table S4.

Figure 8.4. Mean relative abundances of bacterial phyla and genera across diets.



A



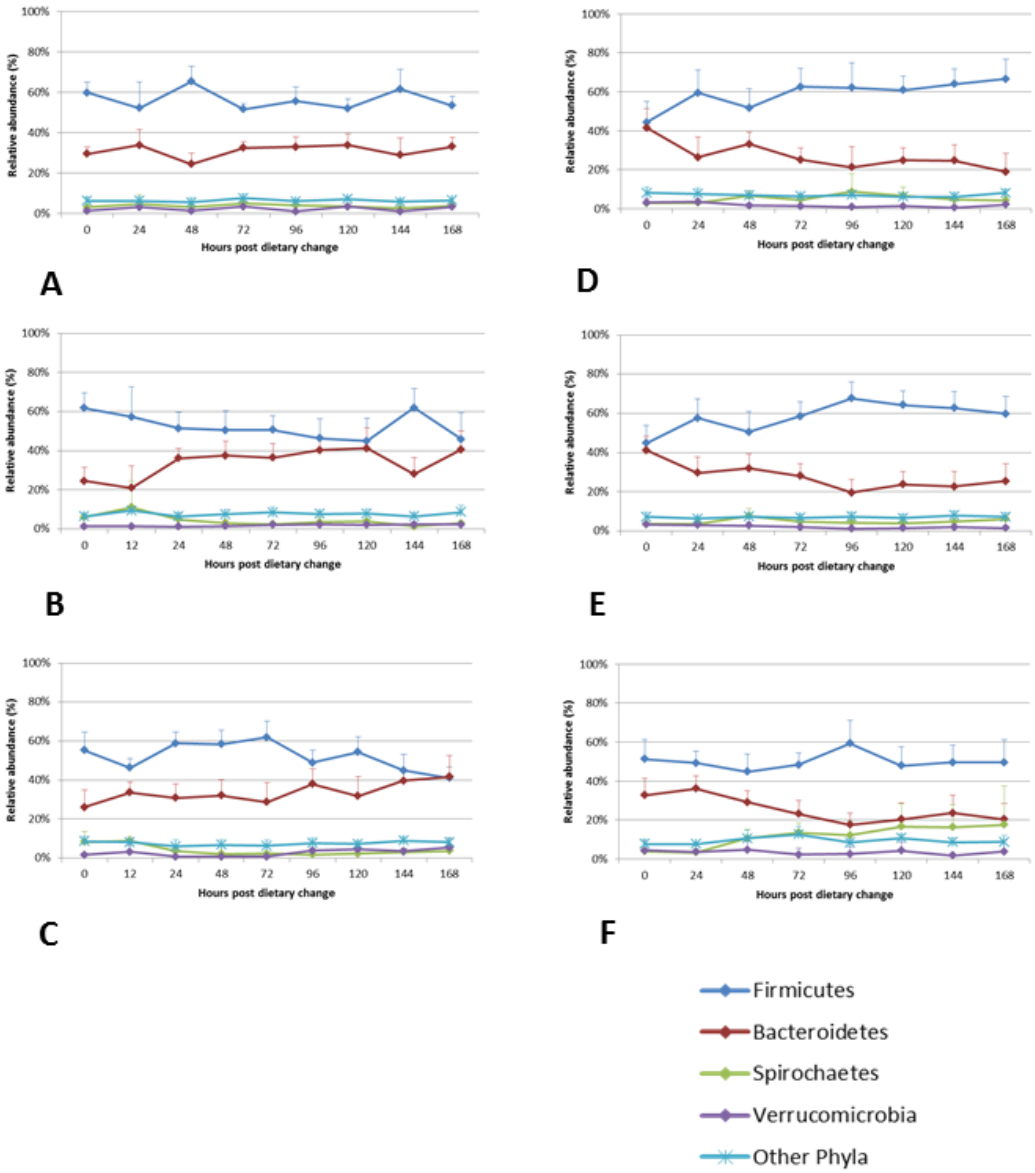
B

Legend: The relative abundances of the four most dominant phyla (Panel A) and six most dominant genera (Panel B) that comprised the faecal bacterial community of horses included in the study are shown in the bar charts. P1, P2, P3 - cut pasture fed during weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage fed with whole oats; and H+O - hay fed with whole oats.

8.4.2.3 Dynamics and stability of the faecal bacterial community following dietary change

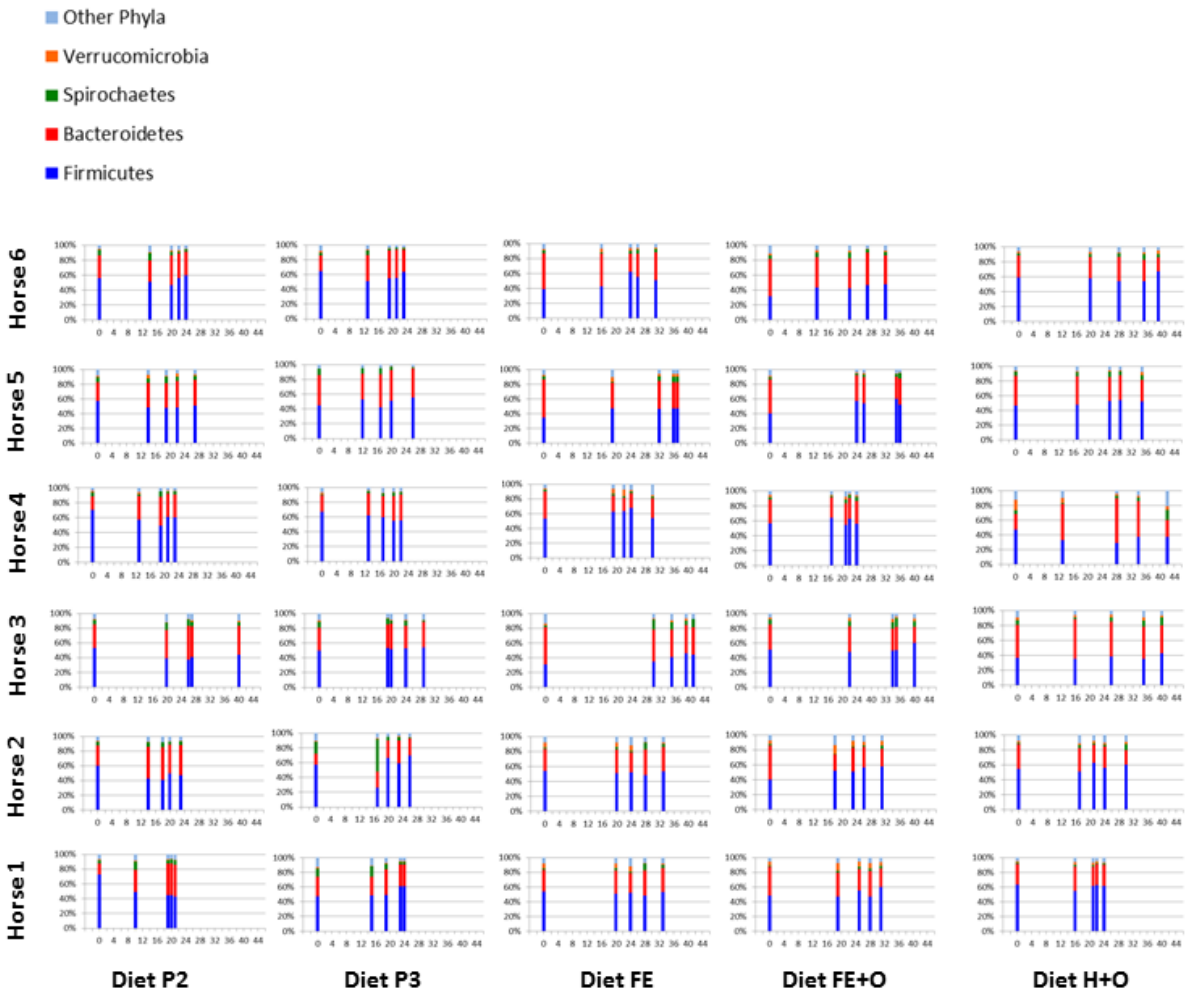
The dynamics of the relative abundances of four most dominant phyla and six most dominant genera, in each diet, are shown in Figures 8.5 and 8.6. Differences in the relative abundances of the dominant phyla were observed as early as time point M1 (when the 1st marker was recovered in the faeces), which ranged between 11-26 h for the diets (19 ± 4 h for Diet P1, 14 ± 3 h for Diet P2, 15 ± 3 h for Diet P3, 21 ± 5 h for Diet FE, 19 ± 4 h for Diet FE+O and 17 ± 2 h for Diet H+O (Figure 8.6, Table 8.3 and Appendix F-8, Figure S3 (Fernandes *et al.*, 2016b)). Across all diets, the faecal bacteria showed differences (0-73%) in relative abundances of Firmicutes and Bacteroidetes within 96 hours following abrupt dietary change (Table 8.4). Changes in the Firmicutes: Bacteroidetes (F:B) ratio were observed from time points T0 to T96, which was a reflection of changes in the relative abundance of the dominant bacterial genera within these two phyla (Table 8.4; Appendix F-7, Figure S2). The diets containing cut pasture (P1, P2 and P3) and hay (H+O) showed some differences (0-45%) in the relative abundance of Firmicutes and Bacteroidetes even after 96 hours following dietary change. There were minor differences (≤5%) in the relative abundances of Firmicutes after the 96-h time point for the diets containing ensiled chopped forage (diets FE and FE+O; Table 8.4 and Figure 8.5). There were no significant differences in the relative abundance at the phylum and genus levels after 96 hours (T120, T144 and T168), when all diets were analysed together (Appendix F-9, Table S5 and Appendix F-10, Table S6).

Figure 8.5. Comparison of the relative abundance of the four most dominant faecal bacterial phyla from 0 to 168 hours following dietary change.



Legend: Mean relative abundances of the four most dominant phyla that comprise the faecal bacterial community of horses included in the study are shown in the line graphs. Each panel represents a dietary treatment block. Panel A) Diet P1- cut pasture; Panel B) Diet P2 – cut pasture; Panel C) Diet P3 – cut pasture; Panel D) Diet FE - ensiled chopped forage; Panel E) Diet FE+O - ensiled chopped forage fed with whole oats; Panel F) Diet H+O - hay fed with whole oats. The time points in hours following dietary change are shown on the x-axis of the primary graph in each panel. Error bars represent the standard deviation (only positive shown).

Figure 8.6. Comparison of the four most dominant phyla with transit time of digesta (T0, M1, M25, M50 and M75) in horses (n=6) across the forage-based diets included in the study.



Legend: Relative abundances of the four most dominant phyla and remaining phyla (Other Phyla) that comprise the faecal bacterial community of horses included in the study are shown in the stacked bar graphs. The sequence of the stacked bars along the x-axis in each panel is: T0 (time 0 when the diet was changed); M1 (time when the first intestinal marker was retrieved in the faeces); M25 (time when 25% of the intestinal markers were retrieved in the faeces); M50 (time when 50% of the intestinal markers were retrieved in the faeces); and M75 (time when 75% of the intestinal markers were retrieved in the faeces). The time points in hours following dietary change are shown on the x-axis of each graph panel and the relative abundance of the phyla as percentage are shown on the y-axis. The diets are labelled as Diet P2 – cut pasture fed during washout period 2; Diet P3 – cut pasture fed during washout period 3; Diet FE- ensiled chopped forage; Diet FE+O - ensiled chopped forage fed with whole oats; and Diet H+O hay fed with whole oats.

Table 8.3. P-values for differences between the most dominant phyla across time points.

Diets	Phyla	Friedman's test (Marker time points)	Post-hoc analysis (T0 vs. M1)	Friedman's test (Day time points)	Post-hoc analysis (T0 vs. T24)
P2	Firmicutes	0.004**	0.046*	0.012**	0.077
	Bacteroidetes	0.010**	0.077	0.019*	0.046*
	Spirochaetes	0.101	0.323	0.007**	0.231
	Verrucomicrobia	0.159	0.662	0.039*	0.446
	Other Phyla	0.456	0.836	0.024*	0.695
P3	Firmicutes	0.701	0.569	0.023*	0.569
	Bacteroidetes	0.161	0.323	0.197	0.446
	Spirochaetes	0.012*	0.230	0.019*	0.169
	Verrucomicrobia	0.180	0.077	0.004**	0.077
	Other Phyla	0.491	0.001**	0.003**	0.058
FE	Firmicutes	0.207	0.077	0.030*	0.077
	Bacteroidetes	0.091	0.107	0.073	0.169
	Spirochaetes	0.091	1.000	0.009**	0.446
	Verrucomicrobia	0.333	0.446	0.082	0.846
	Other Phyla	0.339	0.438	0.014**	0.762
FE+O	Firmicutes	0.228	0.169	0.009**	0.046*
	Bacteroidetes	0.029*	0.077	0.006**	0.046*
	Spirochaetes	0.077	1.000	0.048*	1.000
	Verrucomicrobia	0.091	0.323	0.038*	0.692
	Other Phyla	0.109	0.633	0.218	0.321
H+O	Firmicutes	0.334	0.846	0.066	0.846
	Bacteroidetes	0.034*	0.446	0.006**	0.323
	Spirochaetes	0.643	0.631	0.003**	0.569
	Verrucomicrobia	0.692	1.000	0.487	1.000
	Other Phyla	0.242	0.554	0.025*	0.194

Legend: ** Level of significance $P \leq 0.01$ after Bonferroni correction for multiple comparisons. * indicates trends for a significant difference $P \leq 0.05$. Post-hoc analysis was conducted using the Wilcoxon Signed-rank test. T0 is the time point when the diet was changed, T24 represent the time point 24 hours following dietary change, M1 represent the time point when the first intestinal marker was retrieved in the faeces.

Table 8.4. Difference in the percentage of mean relative abundance of the two most dominant bacterial phyla from 0 to 168 hours post dietary change.

		Firmicutes			Bacteroidetes			
	Time point (h)	Mean	SD	% Difference	Mean	SD	% Difference	F:B ratio
Diet P1	0	0.598	0.053		0.295	0.033		2.03
	24	0.522	0.129	-13%	0.339	0.075	15%	1.54
	48	0.653	0.076	25%	0.245	0.053	-28%	2.67
	72	0.516	0.028	-21%	0.325	0.031	33%	1.59
	96	0.557	0.070	8%	0.330	0.051	2%	1.69
	120	0.520	0.049	-7%	0.339	0.056	3%	1.54
	144	0.616	0.099	18%	0.290	0.083	-14%	2.12
	168	0.534	0.045	-13%	0.331	0.045	14%	1.62
Diet P2	0	0.617	0.080		0.245	0.069		2.52
	12	0.573	0.155	-7%	0.209	0.112	-15%	2.76
	24	0.514	0.084	-10%	0.361	0.050	73%	1.43
	48	0.505	0.099	-2%	0.375	0.073	4%	1.35
	72	0.506	0.072	0%	0.364	0.072	-3%	1.39
	96	0.463	0.099	-8%	0.403	0.071	11%	1.15
	120	0.450	0.117	-3%	0.412	0.104	2%	1.09
	144	0.619	0.098	38%	0.280	0.086	-32%	2.21
	168	0.458	0.136	-26%	0.406	0.096	45%	1.13
Diet P3	0	0.553	0.094		0.261	0.089		2.12
	12	0.463	0.047	-16%	0.337	0.052	29%	1.38
	24	0.589	0.058	27%	0.309	0.071	-8%	1.91
	48	0.583	0.072	-1%	0.321	0.082	4%	1.82
	72	0.620	0.083	6%	0.287	0.099	-11%	2.17
	96	0.489	0.065	-21%	0.379	0.080	32%	1.29
	120	0.544	0.077	11%	0.318	0.101	-16%	1.71
	144	0.449	0.083	-17%	0.397	0.059	25%	1.13
	168	0.411	0.057	-8%	0.417	0.109	5%	0.99
Diet FE	0	0.443	0.107		0.415	0.099		1.07
	24	0.594	0.116	34%	0.264	0.103	-36%	2.25
	48	0.517	0.099	-13%	0.332	0.062	26%	1.56
	72	0.626	0.094	21%	0.251	0.062	-24%	2.50
	96	0.621	0.128	-1%	0.212	0.108	-16%	2.94
	120	0.609	0.072	**-2%**	0.249	0.063	17%	2.45
	144	0.640	0.079	**5%**	0.246	0.081	-1%	2.60
	168	0.666	0.101	**4%**	0.190	0.094	-23%	3.52
Diet FE+O	0	0.448	0.091		0.412	0.072		1.09
	24	0.576	0.097	29%	0.296	0.080	-28%	1.95
	48	0.505	0.104	-12%	0.319	0.074	8%	1.59
	72	0.585	0.073	16%	0.281	0.063	-12%	2.08
	96	0.677	0.083	16%	0.195	0.068	-31%	3.48
	120	0.643	0.070	**-5%**	0.237	0.067	22%	2.71
	144	0.627	0.085	**-2%**	0.227	0.077	-4%	2.76
	168	0.597	0.091	**-5%**	0.255	0.089	12%	2.35
Diet H+O	0	0.513	0.099		0.327	0.086		1.57
	24	0.493	0.061	-4%	0.361	0.067	10%	1.37
	48	0.448	0.091	-9%	0.292	0.059	-19%	1.54
	72	0.484	0.060	8%	0.232	0.069	-21%	2.09
	96	0.593	0.117	23%	0.175	0.060	-25%	3.40
	120	0.479	0.096	-19%	0.204	0.085	17%	2.35
	144	0.496	0.087	4%	0.236	0.092	16%	2.10
	168	0.495	0.117	0%	0.204	0.081	-14%	2.43

Legend: The percentage difference was calculated by (x-y/x) and negative values indicate a percentage decrease. F: B is the Firmicutes: Bacteroidetes ratio. Diets are represented as P1, P2, P3 - cut pasture fed during weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage fed with whole oats; and H+O - hay fed with whole oats.

8.5 Discussion

The present study was designed to evaluate the impact of an abrupt dietary change from pasture to three conserved forage-based diets on the equine hindgut microbiota. This was assessed by intensively measuring the diversity and community structure of the faecal bacteria based on 16S rRNA gene sequencing. This study demonstrated significant differences in the diversity and community structure of faecal bacteria across diets, and supported our hypothesis of an association between the rate of change in the faecal microbiota and the transit time of digesta following dietary transition. Despite the high individual variability in the data, changes in the community structure were observed as early as the appearance of the first intestinal marker in the faeces, and these changes appeared to stabilise 96 hours after the start of the feeding of the treatment diets, indicating resilience of the faecal bacteria following dietary transition. Resilience of an ecological system has previously been defined as the amount of stress or perturbation that a system can tolerate before its trajectory changes towards a different equilibrium state (Folke *et al.*, 2004, Lozupone *et al.*, 2012), or the capacity of a system to absorb disturbance and reorganise while undergoing change so as to still retain essentially the same function, structure, identity, and feedbacks (Walker *et al.*, 2004).

Firmicutes and Bacteroidetes dominated the faecal bacterial profiles of healthy horses in the present study, as previously reported in our work (Fernandes *et al.*, 2014, Fernandes *et al.*, 2016a) and several other equine microbiota studies (Daly *et al.*, 2012, Dougal *et al.*, 2013, Schoster *et al.*, 2013, Costa *et al.*, 2015a). Members of the phyla Firmicutes and Bacteroidetes are anaerobic cellulolytic bacteria associated with fibre digestion, and many of the bacterial genera within these phyla thrive on fermentable carbohydrates (Flint and Bayer, 2008). Although the relative abundance of these dominant phyla significantly differed across all diets in the present study, the domination of Firmicutes, particularly by genera within the families Lachnospiraceae and Ruminococcaceae, and the order Clostridiales, appeared to be associated with the high forage component of the diets. Furthermore, bacterial genera within the families Veillonellaceae, Streptococcaceae and Lactobacillaceae (all Firmicutes) that are commonly

associated with carbohydrate induced laminitis were present at very low abundances (<1%) (Biddle *et al.*, 2013, Moreau *et al.*, 2014). These observations reinforce the importance of maintaining a high proportion of forage in the diet, especially for intensively managed horses.

A diet low in nutrient density (such as forage) is associated with a higher level of both diversity and temporal stability of the microbiota than a diet high in nutrient density (such as diets containing grain) (Hansen *et al.*, 2015). Shifts towards lower or higher F:B ratios have been reported previously in horses fed high proportions of rapidly fermentable carbohydrates (concentrates) (de Fombelle *et al.*, 2001), or in diseased states such as colitis (Costa *et al.*, 2012), large colon volvulus (Weese *et al.*, 2015), carbohydrate induced acute laminitis (Moreau *et al.*, 2014), equine grass sickness (Leng *et al.*, 2015), gastric ulcers (Dong *et al.*, 2016), and metabolic syndrome (Elzinga *et al.*, 2016). This instability in F:B ratios, with a compensatory increase or decrease in the abundance of other phyla such as Verrucomicrobia and Proteobacteria, and associated reduction in diversity, concurred with the general ecological theories that suggest a destabilising effect on the microbial ecosystem (Hansen *et al.*, 2015). The wide range of F:B ratios (~ 1 to 3.5) observed in the healthy horses fed forage-based diets in the present study, demonstrates the elasticity of the microbiota population and our lack of understanding of what may comprise an ideal faecal microbiota profile in healthy horses. While there appears to be some allowance for fluctuations in the microbiota abundances within a healthy hindgut ecosystem, maintaining a high diversity and balance between dominant members of the microbial community may be important for the health and function of this ecosystem.

The present study established that the faecal bacterial community was diet specific, and variation between diets was greater than the variation between horses, which was in agreement with our previous work (Fernandes *et al.*, 2014, Fernandes *et al.*, 2016a). The yearling and mature adult horses that were grazing on pasture in the previous studies (Fernandes *et al.*, 2014, Fernandes *et al.*, 2016a), appeared to have a higher median percentage of Firmicutes (~61-68% vs. ~50-55%) and a higher mean F:B ratio (2.5 vs. 1.4), when compared to adult mares in the present study that were confined in stables and fed freshly cut pasture of similar nutrient composition. These differences in community structure could potentially be due to differences in the age (yearling vs. mature adults) (Biagi *et al.*, 2012, Dougal *et al.*, 2014, Costa *et al.*, 2015b), or due to the management (grazing vs. stabling) of the horses.

In the present study, separation of samples into clusters was observed between the pasture diet groups PP (grazing) and P1, P2 and P3 (stabled) indicating that management of the horses may have influenced the diversity and structure of the faecal bacterial community. The subtle differences in the bacterial community structure of horses fed the cut pasture diets may have been due to changes in the nutrient composition of pasture during the 6-week study period (as previously shown (Fernandes *et al.*, 2016a)). However, the separate clustering of Diets P1 (restricted amounts of cut pasture) from Diets P2 and P3 (*ad libitum* cut pasture), appeared to be associated with differences in the feeding behaviour, dry matter intakes and/or transit times of digesta in the horses (Fernandes *et al.*, 2016b). Differences in the type of feed and the passage rate of digesta may influence the retention time available for microbial fermentation in the hindgut (Drogoul *et al.*, 2001, Fernandes *et al.*, 2016b), thereby influencing the diversity of the faecal bacterial community. Nevertheless, the relationship between diet composition, feeding behaviour, retention of feeds in the GI tract, and their effects on the faecal microbiota and the behavioural stress responses in horses are still poorly understood (Bulmer *et al.*, 2015, Destrez *et al.*, 2015, Flint *et al.*, 2015, Grimm *et al.*, 2016, Julliand and Grimm, 2016), and warrant further investigation.

The hourly collection of faecal matter in the present study enabled the accurate selection of sub-samples to represent specific time points following the event of dietary transition and the retrieval of intestinal markers during the trial. While faecal samples may not directly represent the changes occurring in the caecum, previous studies have shown a high degree of similarity between the bacterial community of faeces and distal compartments of the hindgut (colon) (Blackmore *et al.*, 2013, Schoster *et al.*, 2013, Costa *et al.*, 2015a). The colon has a significant role in fibre digestion and absorption of nutrients, and it is the most common site for digestive disturbances (de Fombelle *et al.*, 2001, de Fombelle *et al.*, 2003). Therefore, the changes in bacterial diversity and community structure observed in the faecal samples in the present study may be a good proxy to similar changes occurring in the colon.

A limitation of the present study was the shorter than expected length of sequences generated by the 2 × 300 base paired-end sequencing protocol on the Illumina MiSeq platform, primarily due to the poor quality of the reverse read sequences. This may have affected the resolution of assignments at higher taxonomic levels (Clarridge, 2004). Nevertheless, the major phyla and genera identified in the present study were similar to previous results (Fernandes *et al.*, 2014, Fernandes *et al.*, 2016a), which indicated that the superior sequence quality of the forward reads that were selected for further downstream analysis, had produced

sufficient taxonomic resolution to validate comparisons between treatment groups of the present study.

The study protocol controlled for several confounding factors (such as variation in the environment, grazing behaviour, gender, age and breed, feeding and management, and nutrient composition of the diets), which appeared to improve the robustness of the study when compared to previous (uncontrolled) experiments (Costa *et al.*, 2012, O' Donnell *et al.*, 2013). The diets in the present study represented common feeding and management practices of horses in New Zealand. The pasture used comprised the most common grass and legume species available for grazing horses in New Zealand, and the ensiled chopped forage, perennial ryegrass hay and whole oats were previously reported as the most commonly fed supplementary feeds for leisure horses and ponies in New Zealand (Fernandes *et al., 2014*). Hence, the results reported on the feed composition and faecal microbiota profiles in the present study may be considered representative of the equine population in New Zealand. However, the variation between individual horses, sources of feed, and management practices must be considered when extrapolating these results further.

The inter-horse variation in the alpha and beta diversities of the bacterial community of horses included in the present study, was smaller than that reported in other (uncontrolled) experiments (Blackmore *et al.*, 2013, Dougal *et al.*, 2013, O' Donnell *et al.*, 2013), indicating that our experimental design minimised the variation observed between horses. Some of the variation observed between the six horses in the present study could be attributed to differences in the feeding behaviour of individual horses, as evidenced by differences in the dry matter intakes (some horses ate more diet or consumed diet faster than other horses), and mean retention times of digesta in the GI tract (Fernandes *et al.*, 2016b). There may be an association between the transit time of digesta and the inter-horse variation observed in the faecal microbiota of horses, however, these factors were beyond the scope of the present study design, and require further investigation with larger numbers of animals.

The diets containing ensiled chopped forage (FE and FE+O) showed the highest F:B ratio (with the highest percentage of unclassified genera identified within the Lachnospiraceae family) and lowest abundance of Verrucomicrobia (a phylum reported to be over represented in horses with chronic laminitis (Steelman *et al.*, 2012)). The nutrient composition profile of the ensiled chopped forage was consistent across time points, when compared to pasture and hay (Fernandes *et al.*, 2016b), and this stability in nutrient composition may have resulted in

a more stable microbiota profile after 96 hours following dietary transition. Although all the horses were healthy during the six dietary periods in the present study, the faecal microbiota profiles of horses consuming the diets containing ensiled chopped forage appeared to be more stable than the microbiota profile of horses consuming the cut pasture or hay diets. This stability in microbiota was perhaps due to the maintenance of a higher F: B ratio than pasture and hay, with very low relative abundances of potentially pathogenic species belonging to the families Veillonellaceae, Streptococcaceae and Lactobacillaceae. However, the microbiota stability was measured over a limited period in the present study (168 hours), and it is possible that the true abundance of pathogenic taxa were not accurately detected in the faeces, when compared to the abundance previously reported in caecal fluid (Moreau *et al.,* 2014). Therefore, some caution is warranted, and further investigation may be required to evaluate the stability of the faecal microbiota in horses fed a consistent diet over a prolonged period.

Spirochaetes are symbiotic microbes involved in digestion of dietary fibre in ruminants (Lee *et al.*, 2013), and have previously been reported in various compartments of the GI tract of horses that were fed hay (Steelman *et al.*, 2012, Costa *et al.*, 2015a). These chemoheterotrophic anaerobic bacteria typically multiply in the presence of acetate, which is produced by microbial fermentation of fibre, suggesting that a forage diet producing large amounts of VFAs could result in an increased abundance of Spirochaetes. The presence of this dietary substrate in the hindgut may explain the increased abundance in members of the Spirochaetes genus *Treponema* observed in the faeces of horses consuming hay in the present study. However, the lower abundance of Spirochaetes in the diets containing cut pasture and ensiled chopped forage when compared to hay remains unexplained, but may be associated with the type of forage, method of preservation, the dry matter and nutrient content, or the increased digesta transit time when compared to hay. The over representation of Spirochaetes in horses fed hay compared to other forages requires further investigation.

Despite forage being the major component of the diet, a rapid and sensitive response to abrupt dietary change was observed in the faecal microbiota of horses in the present study, which is similar to studies where horses were transitioned from ensiled chopped forage to pasture (Fernandes *et al.*, 2014) or between two types of hay (Grimm *et al.*, 2016). Abrupt dietary transition affected the GI microbiota of the horses, with changes occurring as soon as the new diet transits the GI tract, without apparent negative effects on the health of the horses. Horses appear to have a resident core hindgut microbiota (Blackmore *et al.*, 2013, Dougal *et al.*, 2013), and evidence that this resident bacterial community responds rapidly to dietary

change (within hours), represents the evolutionary ecological niche of the horse as an opportunistic browser, susceptible to frequent dietary change (Janis, 1976). The digestive system of the horse is adapted to optimise a high-fibre diet consumed in small amounts over prolonged periods, and horses appear to be evolutionarily driven to tolerate changes in dietary substrates, as long as forage is the major component of the diet (Frape, 2010). However, the physiological limitations of this adaptability of the hindgut microbiota to rapid change need to be considered during intensive management of horses. The feeding of high-concentrate diets, low forage quality and quantity, meal feeding, and confinement housing all present unique challenges to the equine hindgut microbiome and are important risk factors associated with GI disturbances that can impair the performance of horses (Cohen and Peloso, 1996, Cohen *et al.*, 1999).

The equine hindgut microbiota is a complex ecosystem that appears to be influenced by multiple intrinsic and extrinsic factors, and the function and stability of this complex ecosystem may be integrated by equally complex drivers and feedback loops (Cramer *et al.*, 2011). Over the last decade, the use of molecular sequencing techniques has rapidly progressed our understanding of this hindgut ecosystem, with many studies focussing on the population dynamics of the faecal microbiota of healthy horses in response to extrinsic factors (such as changes due to dietary management or disease) (Daly *et al.*, 2001, Costa *et al.*, 2012, Dougal *et al.*, 2013, Fernandes *et al.*, 2014). However, there is great variation in the data on what comprises a healthy equine hindgut microbiota profile. Further investigations using functional genomics, metabolomics, and a complex systems approach are required to understand the function and stability of these anaerobic as yet uncultured bacteria, and their interactions within this complex ecosystem.

8.6 Conclusion

The present study supported the hypothesis that the diversity and community structure of the faecal bacteria of horses are diet-specific, and the population dynamics showed that the bacterial community was resilient following dietary transition. The faecal bacteria responded rapidly to dietary change, and appeared to be associated with the transit time of digesta through the GI tract. Competition horses often experience major abrupt dietary and management changes around the time of training and competitive events. Therefore, the findings of this study have implications for developing feeding strategies, which benefit intensively managed

horses that are predisposed to, or recovering from, GI disturbances, and reinforces the ecological niche of horses, where forage, and large proportions of it, comprise the major part of a horses' diet.

8.7 Acknowledgments

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Appendix F

This appendix provides supplementary information on the tables and figures cited in Chapter 8.

F-1. An illustration of the faecal sample collection protocol.

F-2. Table S1. Metadata on the faecal samples (n=377) included in the study on the population dynamics of faecal microbiota of forage-fed horses (n=6).

F-3. Table S2. Diet-wise comparison of the alpha diversity indices of faecal bacterial genera across the three time points on days 5 (120h), 6 (144h) and 7 (168h) of the study period.

F-4. Figure S1. Principal coordinate analysis of the faecal bacterial communities in samples obtained from horses fed pasture (P1, P2, P3) and three conserved forage diets (FE, FE+O, H+O) on days 5, 6 and 7 of the study period.

F-5. Table S3. Relative abundance of the bacterial phyla in the faeces compared across diets.

F-6. Table S4. Relative abundance of the bacterial genera in the faeces compared across diets.

F-7. Figure S2. Comparison of the relative abundances of the six dominant faecal bacterial genera from 0 to 168 hours following dietary change.

F-8. Figure S3. Comparson of the six most dominant genera with transit time of digesta (T0, M1, M25, M50 and M75) in horses (n=6) across the forage-based diets included in the study.

F-9. Table S5. Relative abundance of faecal bacterial phyla across three time points on day 5 (T120), day 6 (T144) and day 7 (T168) of the study period.

F-10. Table S6. Relative abundance of faecal bacterial genera across three time points on day 5 (T120), day 6 (T144) and day 7 (T168) of the study period.

F-1. An illustration of the faecal sample collection protocol.


(A) Collection of faecal matter from bedding material in stables


(B) Collection of a sub-sample of faecal matter into cryogenic vials

(C) Cryofreezing in liquid nitrogen … …

(D) Sample collection equipment

F-2. Table S1. Metadata on the faecal samples (n=377) included in the study on the population dynamics of faecal microbiota of forage-fed horses (n=6).

SampleID	Barcode1	Barcode2	MiSeqRun	SampleDescription	Horse	TimePoint	Week	Diet	Age
1BLOCK1-A1H1T0_W0PP	CGAGAGTT	ATCGTACG	1	H1T0_W0PP	H1	T0	W0	PP	9
2BLOCK1-A2H2T0_W0PP	GACATAGT	ATCGTACG	1	H2T0_W0PP	H2	T0	W0	PP	16
3BLOCK1-A3H3T0_W0PP	ACGCTACT	ATCGTACG	1	H3T0_W0PP	H3	T0	W0	PP	16
4BLOCK1-A4H4T0_W0PP	ACTCACTG	ATCGTACG	1	H4T0_W0PP	H4	T0	W0	PP	16
5BLOCK1-A5H5T0_W0PP	TGAGTACG	ATCGTACG	1	H5T0_W0PP	H5	T0	W0	PP	16
6BLOCK1-A6H6T0_W0PP	CTGCGTAG	ATCGTACG	1	H6T0_W0PP	H6	T0	W0	PP	9
7BLOCK1-A7H1T24_W1P	TAGTCTCC	ATCGTACG	1	H1T24_W1P	H1	T24	W1	P1	9
8BLOCK1-A8H2T24_W1P	CGAGCGAC	ATCGTACG	1	H2T24_W1P	H2	T24	W1	P1	16
9BLOCK1-A9H3T24_W1P	ACTACGAC	ATCGTACG	1	H3T24_W1P	H3	T24	W1	P1	16
10BLOCK1-A10H4T24_W1P	GTCTGCTA	ATCGTACG	1	H4T24_W1P	H4	T24	W1	P1	16
11BLOCK1-A11H5T24_W1P	GTCTATGA	ATCGTACG	1	H5T24_W1P	H5	T24	W1	P1	16
12BLOCK1-A12H6T24_W1P	TATAGCGA	ATCGTACG	1	H6T24_W1P	H6	T24	W1	P1	9
13BLOCK1-B1H1T48_W1P	CGAGAGTT	ACTATCTG	1	H1T48_W1P	H1	T48	W1	P1	9
14BLOCK1-B2H2T48_W1P	GACATAGT	ACTATCTG	1	H2T48_W1P	H2	T48	W1	P1	16
15BLOCK1-B3H3T48_W1P	ACGCTACT	ACTATCTG	1	H3T48_W1P	H3	T48	W1	P1	16
16BLOCK1-B4H4T48_W1P	ACTCACTG	ACTATCTG	1	H4T48_W1P	H4	T48	W1	P1	16
17BLOCK1-B5H5T48_W1P	TGAGTACG	ACTATCTG	1	H5T48_W1P	H5	T48	W1	P1	16
18BLOCK1-B6H6T48_W1P	CTGCGTAG	ACTATCTG	1	H6T48_W1P	H6	T48	W1	P1	9
19BLOCK1-B7H1T72_W1P	TAGTCTCC	ACTATCTG	1	H1T72_W1P	H1	T72	W1	P1	9
20BLOCK1-B8H2T72_W1P	CGAGCGAC	ACTATCTG	1	H2T72_W1P	H2	T72	W1	P1	16
21BLOCK1-B9H3T72_W1P	ACTACGAC	ACTATCTG	1	H3T72_W1P	H3	T72	W1	P1	16
22BLOCK1-B10H4T72_W1P	GTCTGCTA	ACTATCTG	1	H4T72_W1P	H4	T72	W1	P1	16
23BLOCK1-B11H5T72_W1P	GTCTATGA	ACTATCTG	1	H5T72_W1P	H5	T72	W1	P1	16
24BLOCK1-B12H6T72_W1P	TATAGCGA	ACTATCTG	1	H6T72_W1P	H6	T72	W1	P1	9
25BLOCK1-C1H1T96_W1P	CGAGAGTT	TAGCGAGT	1	H1T96_W1P	H1	T96	W1	P1	9
26BLOCK1-C2H2T96_W1P	GACATAGT	TAGCGAGT	1	H2T96_W1P	H2	T96	W1	P1	16
27BLOCK1-C3H3T96_W1P	ACGCTACT	TAGCGAGT	1	H3T96_W1P	H3	T96	W1	P1	16
28BLOCK1-C4H4T96_W1P	ACTCACTG	TAGCGAGT	1	H4T96_W1P	H4	T96	W1	P1	16
29BLOCK1-C5H5T96_W1P	TGAGTACG	TAGCGAGT	1	H5T96_W1P	H5	T96	W1	P1	16
30BLOCK1-C6H6T96_W1P	CTGCGTAG	TAGCGAGT	1	H6T96_W1P	H6	T96	W1	P1	9

31BLOCK1-C7H1T120_W1P	TAGTCTCC	TAGCGAGT	1	H1T120_W1P	H1	T120	W1	P1	9
32BLOCK1-C8H2T120_W1P	CGAGCGAC	TAGCGAGT	1	H2T120_W1P	H2	T120	W1	P1	16
33BLOCK1-C9H3T120_W1P	ACTACGAC	TAGCGAGT	1	H3T120_W1P	H3	T120	W1	P1	16
34BLOCK1-C10H4T120_W1P	GTCTGCTA	TAGCGAGT	1	H4T120_W1P	H4	T120	W1	P1	16
35BLOCK1-C11H5T120_W1P	GTCTATGA	TAGCGAGT	1	H5T120_W1P	H5	T120	W1	P1	16
36BLOCK1-C12H6T120_W1P	TATAGCGA	TAGCGAGT	1	H6T120_W1P	H6	T120	W1	P1	9
37BLOCK1-D1H1T144_W1P	CGAGAGTT	CTGCGTGT	1	H1T144_W1P	H1	T144	W1	P1	9
38BLOCK1-D2H2T144_W1P	GACATAGT	CTGCGTGT	1	H2T144_W1P	H2	T144	W1	P1	16
39BLOCK1-D3H3T144_W1P	ACGCTACT	CTGCGTGT	1	H3T144_W1P	H3	T144	W1	P1	16
40BLOCK1-D4H4T144_W1P	ACTCACTG	CTGCGTGT	1	H4T144_W1P	H4	T144	W1	P1	16
41BLOCK1-D5H5T144_W1P	TGAGTACG	CTGCGTGT	1	H5T144_W1P	H5	T144	W1	P1	16
42BLOCK1-D6H6T144_W1P	CTGCGTAG	CTGCGTGT	1	H6T144_W1P	H6	T144	W1	P1	9
43BLOCK1-D7H1T168_W1P	TAGTCTCC	CTGCGTGT	1	H1T168_W1P	H1	T168	W1	P1	9
44BLOCK1-D8H2T168_W1P	CGAGCGAC	CTGCGTGT	1	H2T168_W1P	H2	T168	W1	P1	16
45BLOCK1-D9H3T168_W1P	ACTACGAC	CTGCGTGT	1	H3T168_W1P	H3	T168	W1	P1	16
46BLOCK1-D10H4T168_W1P	GTCTGCTA	CTGCGTGT	1	H4T168_W1P	H4	T168	W1	P1	16
47BLOCK1-D11H5T168_W1P	GTCTATGA	CTGCGTGT	1	H5T168_W1P	H5	T168	W1	P1	16
48BLOCK1-D12H6T168_W1P	TATAGCGA	CTGCGTGT	1	H6T168_W1P	H6	T168	W1	P1	9
49BLOCK1-E1H1M1_W2FE	CGAGAGTT	TCATCGAG	1	H1M1_W2FE	H1	M1	W2	FE	9
50BLOCK1-E2H2M1_W2FE	GACATAGT	TCATCGAG	1	H2M1_W2FE	H2	M1	W2	FE	16
51BLOCK1-E3H3M1_W2FEO	ACGCTACT	TCATCGAG	1	H3M1_W2FEO	H3	M1	W2	FEO	16
52BLOCK1-E4H4M1_W2FEO	ACTCACTG	TCATCGAG	1	H4M1_W2FEO	H4	M1	W2	FEO	16
53BLOCK1-E5H5M1_W2HO	TGAGTACG	TCATCGAG	1	H5M1_W2HO	H5	M1	W2	HO	16
54BLOCK1-E6H6M1_W2HO	CTGCGTAG	TCATCGAG	1	H6M1_W2HO	H6	M1	W2	HO	9
55BLOCK1-E7H1M25_W2FE	TAGTCTCC	TCATCGAG	1	H1M25_W2FE	H1	M25	W2	FE	9
56BLOCK1-E8H2M25_W2FE	CGAGCGAC	TCATCGAG	1	H2M25_W2FE	H2	M25	W2	FE	16
57BLOCK1-E9H3M25_W2FEO	ACTACGAC	TCATCGAG	1	H3M25_W2FEO	H3	M25	W2	FEO	16
58BLOCK1-E10H4M25_W2FEO	GTCTGCTA	TCATCGAG	1	H4M25_W2FEO	H4	M25	W2	FEO	16
59BLOCK1-E11H5M25_W2HO	GTCTATGA	TCATCGAG	1	H5M25_W2HO	H5	M25	W2	HO	16
60BLOCK1-E12H6M25_W2HO	TATAGCGA	TCATCGAG	1	H6M25_W2HO	H6	M25	W2	HO	9
61BLOCK1-F1H1M50_W2FE	CGAGAGTT	CGTGAGTG	1	H1M50_W2FE	H1	M50	W2	FE	9
62BLOCK1-F2H2M50_W2FE	GACATAGT	CGTGAGTG	1	H2M50_W2FE	H2	M50	W2	FE	16
63BLOCK1-F3H3M50_W2FEO	ACGCTACT	CGTGAGTG	1	H3M50_W2FEO	H3	M50	W2	FEO	16
64BLOCK1-F4H4M50_W2FEO	ACTCACTG	CGTGAGTG	1	H4M50_W2FEO	H4	M50	W2	FEO	16
65BLOCK1-F5H5M50_W2HO	TGAGTACG	CGTGAGTG	1	H5M50_W2HO	H5	M50	W2	HO	16

66BLOCK1-F6H6M50_W2HO	CTGCGTAG	CGTGAGTG	1	H6M50_W2HO	H6	M50	W2	HO	9
67BLOCK1-F7H1M75_W2FE	TAGTCTCC	CGTGAGTG	1	H1M75_W2FE	H1	M75	W2	FE	9
68BLOCK1-F8H2M75_W2FE	CGAGCGAC	CGTGAGTG	1	H2M75_W2FE	H2	M75	W2	FE	16
69BLOCK1-F9H3M75_W2FEO	ACTACGAC	CGTGAGTG	1	H3M75_W2FEO	H3	M75	W2	FEO	16
70BLOCK1-F10H4M75_W2FEO	GTCTGCTA	CGTGAGTG	1	H4M75_W2FEO	H4	M75	W2	FEO	16
71BLOCK1-F11H5M75_W2HO	GTCTATGA	CGTGAGTG	1	H5M75_W2HO	H5	M75	W2	HO	16
72BLOCK1-F12H6M75_W2HO	TATAGCGA	CGTGAGTG	1	H6M75_W2HO	H6	M75	W2	HO	9
BLANK SAMPLE									
74BLOCK1-G2H2T24_W2FE	GACATAGT	GGATATCT	1	H2T24_W2FE	H2	T24	W2	FE	16
75BLOCK1-G3H3T24_W2FEO	ACGCTACT	GGATATCT	1	H3T24_W2FEO	H3	T24	W2	FEO	16
76BLOCK1-G4H4T24_W2FEO	ACTCACTG	GGATATCT	1	H4T24_W2FEO	H4	T24	W2	FEO	16
77BLOCK1-G5H5T24_W2HO	TGAGTACG	GGATATCT	1	H5T24_W2HO	H5	T24	W2	HO	16
78BLOCK1-G6H6T24_W2HO	CTGCGTAG	GGATATCT	1	H6T24_W2HO	H6	T24	W2	HO	9
79BLOCK1-G7H1T48_W2FE	TAGTCTCC	GGATATCT	1	H1T48_W2FE	H1	T48	W2	FE	9
80BLOCK1-G8H2T48_W2FE	CGAGCGAC	GGATATCT	1	H2T48_W2FE	H2	T48	W2	FE	16
81BLOCK1-G9H3T48_W2FEO	ACTACGAC	GGATATCT	1	H3T48_W2FEO	H3	T48	W2	FEO	16
82BLOCK1-G10H4T48_W2FEO	GTCTGCTA	GGATATCT	1	H4T48_W2FEO	H4	T48	W2	FEO	16
83BLOCK1-G11H5T48_W2HO	GTCTATGA	GGATATCT	1	H5T48_W2HO	H5	T48	W2	HO	16
84BLOCK1-G12H6T48_W2HO	TATAGCGA	GGATATCT	1	H6T48_W2HO	H6	T48	W2	HO	9
85BLOCK1-H1H1T72_W2FE	CGAGAGTT	GACACCGT	1	H1T72_W2FE	H1	T72	W2	FE	9
86BLOCK1-H2H2T72_W2FE	GACATAGT	GACACCGT	1	H2T72_W2FE	H2	T72	W2	FE	16
87BLOCK1-H3H3T72_W2FEO	ACGCTACT	GACACCGT	1	H3T72_W2FEO	H3	T72	W2	FEO	16
88BLOCK1-H4H4T72_W2FEO	ACTCACTG	GACACCGT	1	H4T72_W2FEO	H4	T72	W2	FEO	16
89BLOCK1-H5H5T72_W2HO	TGAGTACG	GACACCGT	1	H5T72_W2HO	H5	T72	W2	HO	16
90BLOCK1-H6H6T72_W2HO	CTGCGTAG	GACACCGT	1	H6T72_W2HO	H6	T72	W2	HO	9
91BLOCK1-H7H1T96_W2FE	TAGTCTCC	GACACCGT	1	H1T96_W2FE	H1	T96	W2	FE	9
92BLOCK1-H8H2T96_W2FE	CGAGCGAC	GACACCGT	1	H2T96_W2FE	H2	T96	W2	FE	16
93BLOCK1-H9H3T96_W2FEO	ACTACGAC	GACACCGT	1	H3T96_W2FEO	H3	T96	W2	FEO	16
94BLOCK1-H10H4T96_W2FEO	GTCTGCTA	GACACCGT	1	H4T96_W2FEO	H4	T96	W2	FEO	16
95BLOCK1-H11H5T96_W2HO	GTCTATGA	GACACCGT	1	H5T96_W2HO	H5	T96	W2	HO	16
96BLOCK1-H12H6T96_W2HO	TATAGCGA	GACACCGT	1	H6T96_W2HO	H6	T96	W2	HO	9
97BLOCK2-A1H1T120_W2FE	CGAGAGTT	CTACTATA	1	H1T120_W2FE	H1	T120	W2	FE	9
98BLOCK2-A2H2T120_W2FE	GACATAGT	CTACTATA	1	H2T120_W2FE	H2	T120	W2	FE	16
99BLOCK2-A3H3T120_W2FEO	ACGCTACT	CTACTATA	1	H3T120_W2FEO	H3	T120	W2	FEO	16
100BLOCK2-A4H4T120_W2FEO	ACTCACTG	CTACTATA	1	H4T120_W2FEO	H4	T120	W2	FEO	16

101BLOCK2-A5H5T120_W2HO	TGAGTACG	CTACTATA	1	H5T120_W2HO	H5	T120	W2	HO	16
102BLOCK2-A6H6T120_W2HO	CTGCGTAG	CTACTATA	1	H6T120_W2HO	H6	T120	W2	HO	9
103BLOCK2-A7H1T144_W2FE	TAGTCTCC	CTACTATA	1	H1T144_W2FE	H1	T144	W2	FE	9
104BLOCK2-A8H2T144_W2FE	CGAGCGAC	CTACTATA	1	H2T144_W2FE	H2	T144	W2	FE	16
105BLOCK2-A9H3T144_W2FEO	ACTACGAC	CTACTATA	1	H3T144_W2FEO	H3	T144	W2	FEO	16
106BLOCK2-A10H4T144_W2FEO	GTCTGCTA	CTACTATA	1	H4T144_W2FEO	H4	T144	W2	FEO	16
107BLOCK2-A11H5T144_W2HO	GTCTATGA	CTACTATA	1	H5T144_W2HO	H5	T144	W2	HO	16
108BLOCK2-A12H6T144_W2HO	TATAGCGA	CTACTATA	1	H6T144_W2HO	H6	T144	W2	HO	9
109BLOCK2-B1H1T168_W2FE	CGAGAGTT	CGTTACTA	1	H1T168_W2FE	H1	T168	W2	FE	9
110BLOCK2-B2H2T168_W2FE	GACATAGT	CGTTACTA	1	H2T168_W2FE	H2	T168	W2	FE	16
111BLOCK2-B3H3T168_W2FEO	ACGCTACT	CGTTACTA	1	H3T168_W2FEO	H3	T168	W2	FEO	16
112BLOCK2-B4H4T168_W2FEO	ACTCACTG	CGTTACTA	1	H4T168_W2FEO	H4	T168	W2	FEO	16
113BLOCK2-B5H5T168_W2HO	TGAGTACG	CGTTACTA	1	H5T168_W2HO	H5	T168	W2	HO	16
114BLOCK2-B6H6T168_W2HO	CTGCGTAG	CGTTACTA	1	H6T168_W2HO	H6	T168	W2	HO	9
115BLOCK2-B7H1M1_W3P	TAGTCTCC	CGTTACTA	1	H1M1_W3P	H1	M1	W3	P2	9
116BLOCK2-B8H2M1_W3P	CGAGCGAC	CGTTACTA	1	H2M1_W3P	H2	M1	W3	P2	16
117BLOCK2-B9H3M1_W3P	ACTACGAC	CGTTACTA	1	H3M1_W3P	H3	M1	W3	P2	16
118BLOCK2-B10H4M1_W3P	GTCTGCTA	CGTTACTA	1	H4M1_W3P	H4	M1	W3	P2	16
119BLOCK2-B11H5M1_W3P	GTCTATGA	CGTTACTA	1	H5M1_W3P	H5	M1	W3	P2	16
120BLOCK2-B12H6M1_W3P	TATAGCGA	CGTTACTA	1	H6M1_W3P	H6	M1	W3	P2	9
121BLOCK2-C1H1M25_W3P	CGAGAGTT	AGAGTCAC	1	H1M25_W3P	H1	M25	W3	P2	9
122BLOCK2-C2H2M25_W3P	GACATAGT	AGAGTCAC	1	H2M25_W3P	H2	M25	W3	P2	16
123BLOCK2-C3H3M25_W3P	ACGCTACT	AGAGTCAC	1	H3M25_W3P	H3	M25	W3	P2	16
124BLOCK2-C4H4M25_W3P	ACTCACTG	AGAGTCAC	1	H4M25_W3P	H4	M25	W3	P2	16
125BLOCK2-C5H5M25_W3P	TGAGTACG	AGAGTCAC	1	H5M25_W3P	H5	M25	W3	P2	16
126BLOCK2-C6H6M25_W3P	CTGCGTAG	AGAGTCAC	1	H6M25_W3P	H6	M25	W3	P2	9
127BLOCK2-C7H1M50_W3P	TAGTCTCC	AGAGTCAC	1	H1M50_W3P	H1	M50	W3	P2	9
128BLOCK2-C8H2M50_W3P	CGAGCGAC	AGAGTCAC	1	H2M50_W3P	H2	M50	W3	P2	16
129BLOCK2-C9H3M50_W3P	ACTACGAC	AGAGTCAC	1	H3M50_W3P	H3	M50	W3	P2	16
130BLOCK2-C10H4M50_W3P	GTCTGCTA	AGAGTCAC	1	H4M50_W3P	H4	M50	W3	P2	16
131BLOCK2-C11H5M50_W3P	GTCTATGA	AGAGTCAC	1	H5M50_W3P	H5	M50	W3	P2	16
132BLOCK2-C12H6M50_W3P	TATAGCGA	AGAGTCAC	1	H6M50_W3P	H6	M50	W3	P2	9
133BLOCK2-D1H1M75_W3P	CGAGAGTT	TACGAGAC	1	H1M75_W3P	H1	M75	W3	P2	9
134BLOCK2-D2H2M75_W3P	GACATAGT	TACGAGAC	1	H2M75_W3P	H2	M75	W3	P2	16
135BLOCK2-D3H3M75_W3P	ACGCTACT	TACGAGAC	1	H3M75_W3P	H3	M75	W3	P2	16

136BLOCK2-D4H4M75_W3P	ACTCACTG	TACGAGAC	1	H4M75_W3P	H4	M75	W3	P2	16
137BLOCK2-D5H5M75_W3P	TGAGTACG	TACGAGAC	1	H5M75_W3P	H5	M75	W3	P2	16
138BLOCK2-D6H6M75_W3P	CTGCGTAG	TACGAGAC	1	H6M75_W3P	H6	M75	W3	P2	9
139BLOCK2-D7H1T24_W3P	TAGTCTCC	TACGAGAC	1	H1T24_W3P	H1	T24	W3	P2	9
140BLOCK2-D8H2T24_W3P	CGAGCGAC	TACGAGAC	1	H2T24_W3P	H2	T24	W3	P2	16
141BLOCK2-D9H3T24_W3P	ACTACGAC	TACGAGAC	1	H3T24_W3P	H3	T24	W3	P2	16
142BLOCK2-D10H4T24_W3P	GTCTGCTA	TACGAGAC	1	H4T24_W3P	H4	T24	W3	P2	16
143BLOCK2-D11H5T24_W3P	GTCTATGA	TACGAGAC	1	H5T24_W3P	H5	T24	W3	P2	16
BLANK SAMPLE									
145BLOCK2-E1H1T48_W3P	CGAGAGTT	ACGTCTCG	1	H1T48_W3P	H1	T48	W3	P2	9
146BLOCK2-E2H2T48_W3P	GACATAGT	ACGTCTCG	1	H2T48_W3P	H2	T48	W3	P2	16
147BLOCK2-E3H3T48_W3P	ACGCTACT	ACGTCTCG	1	H3T48_W3P	H3	T48	W3	P2	16
148BLOCK2-E4H4T48_W3P	ACTCACTG	ACGTCTCG	1	H4T48_W3P	H4	T48	W3	P2	16
149BLOCK2-E5H5T48_W3P	TGAGTACG	ACGTCTCG	1	H5T48_W3P	H5	T48	W3	P2	16
150BLOCK2-E6H6T48_W3P	CTGCGTAG	ACGTCTCG	1	H6T48_W3P	H6	T48	W3	P2	9
151BLOCK2-E7H1T72_W3P	TAGTCTCC	ACGTCTCG	1	H1T72_W3P	H1	T72	W3	P2	9
152BLOCK2-E8H2T72_W3P	CGAGCGAC	ACGTCTCG	1	H2T72_W3P	H2	T72	W3	P2	16
153BLOCK2-E9H3T72_W3P	ACTACGAC	ACGTCTCG	1	H3T72_W3P	H3	T72	W3	P2	16
154BLOCK2-E10H4T72_W3P	GTCTGCTA	ACGTCTCG	1	H4T72_W3P	H4	T72	W3	P2	16
155BLOCK2-E11H5T72_W3P	GTCTATGA	ACGTCTCG	1	H5T72_W3P	H5	T72	W3	P2	16
156BLOCK2-E12H6T72_W3P	TATAGCGA	ACGTCTCG	1	H6T72_W3P	H6	T72	W3	P2	9
157BLOCK2-F1H1T96_W3P	CGAGAGTT	TCGACGAG	1	H1T96_W3P	H1	T96	W3	P2	9
158BLOCK2-F2H2T96_W3P	GACATAGT	TCGACGAG	1	H2T96_W3P	H2	T96	W3	P2	16
159BLOCK2-F3H3T96_W3P	ACGCTACT	TCGACGAG	1	H3T96_W3P	H3	T96	W3	P2	16
160BLOCK2-F4H4T96_W3P	ACTCACTG	TCGACGAG	1	H4T96_W3P	H4	T96	W3	P2	16
161BLOCK2-F5H5T96_W3P	TGAGTACG	TCGACGAG	1	H5T96_W3P	H5	T96	W3	P2	16
162BLOCK2-F6H6T96_W3P	CTGCGTAG	TCGACGAG	1	H6T96_W3P	H6	T96	W3	P2	9
163BLOCK2-F7H1T120_W3P	TAGTCTCC	TCGACGAG	1	H1T120_W3P	H1	T120	W3	P2	9
164BLOCK2-F8H2T120_W3P	CGAGCGAC	TCGACGAG	1	H2T120_W3P	H2	T120	W3	P2	16
165BLOCK2-F9H3T120_W3P	ACTACGAC	TCGACGAG	1	H3T120_W3P	H3	T120	W3	P2	16
166BLOCK2-F10H4T120_W3P	GTCTGCTA	TCGACGAG	1	H4T120_W3P	H4	T120	W3	P2	16
167BLOCK2-F11H5T120_W3P	GTCTATGA	TCGACGAG	1	H5T120_W3P	H5	T120	W3	P2	16
168BLOCK2-F12H6T120_W3P	TATAGCGA	TCGACGAG	1	H6T120_W3P	H6	T120	W3	P2	9
169BLOCK2-G1H1T144_W3P	CGAGAGTT	GATCGTGT	1	H1T144_W3P	H1	T144	W3	P2	9
170BLOCK2-G2H2T144_W3P	GACATAGT	GATCGTGT	1	H2T144_W3P	H2	T144	W3	P2	16

171BLOCK2-G3H3T144_W3P	ACGCTACT	GATCGTGT	1	H3T144_W3P	H3	T144	W3	P2	16
172BLOCK2-G4H4T144_W3P	ACTCACTG	GATCGTGT	1	H4T144_W3P	H4	T144	W3	P2	16
173BLOCK2-G5H5T144_W3P	TGAGTACG	GATCGTGT	1	H5T144_W3P	H5	T144	W3	P2	16
174BLOCK2-G6H6T144_W3P	CTGCGTAG	GATCGTGT	1	H6T144_W3P	H6	T144	W3	P2	9
175BLOCK2-G7H1T168_W3P	TAGTCTCC	GATCGTGT	1	H1T168_W3P	H1	T168	W3	P2	9
176BLOCK2-G8H2T168_W3P	CGAGCGAC	GATCGTGT	1	H2T168_W3P	H2	T168	W3	P2	16
177BLOCK2-G9H3T168_W3P	ACTACGAC	GATCGTGT	1	H3T168_W3P	H3	T168	W3	P2	16
178BLOCK2-G10H4T168_W3P	GTCTGCTA	GATCGTGT	1	H4T168_W3P	H4	T168	W3	P2	16
179BLOCK2-G11H5T168_W3P	GTCTATGA	GATCGTGT	1	H5T168_W3P	H5	T168	W3	P2	16
180BLOCK2-G12H6T168_W3P	TATAGCGA	GATCGTGT	1	H6T168_W3P	H6	T168	W3	P2	9
181BLOCK2-H1H1M1_W4HO	CGAGAGTT	GTCAGATA	1	H1M1_W4HO	H1	M1	W4	HO	9
182BLOCK2-H2H2M1_W4HO	GACATAGT	GTCAGATA	1	H2M1_W4HO	H2	M1	W4	HO	16
183BLOCK2-H3H3M1_W4FE	ACGCTACT	GTCAGATA	1	H3M1_W4FE	H3	M1	W4	FE	16
184BLOCK2-H4H4M1_W4FE	ACTCACTG	GTCAGATA	1	H4M1_W4FE	H4	M1	W4	FE	16
185BLOCK2-H5H5M1_W4FEO	TGAGTACG	GTCAGATA	1	H5M1_W4FEO	H5	M1	W4	FEO	16
186BLOCK2-H6H6M1_W4FEO	CTGCGTAG	GTCAGATA	1	H6M1_W4FEO	H6	M1	W4	FEO	9
187BLOCK2-H7H1M25_W4HO	TAGTCTCC	GTCAGATA	1	H1M25_W4HO	H1	M25	W4	HO	9
188BLOCK2-H8H2M25_W4HO	CGAGCGAC	GTCAGATA	1	H2M25_W4HO	H2	M25	W4	HO	16
189BLOCK2-H9H3M25_W4FE	ACTACGAC	GTCAGATA	1	H3M25_W4FE	H3	M25	W4	FE	16
190BLOCK2-H10H4M25_W4FE	GTCTGCTA	GTCAGATA	1	H4M25_W4FE	H4	M25	W4	FE	16
191BLOCK2-H11H5M25_W4FEO	GTCTATGA	GTCAGATA	1	H5M25_W4FEO	H5	M25	W4	FEO	16
192BLOCK2-H12H6M25_W4FEO	TATAGCGA	GTCAGATA	1	H6M25_W4FEO	H6	M25	W4	FEO	9
193BLOCK3-A1H1M50_W4HO	CTCGACTT	ATCGTACG	2	H1M50_W4HO	H1	M50	W4	HO	9
194BLOCK3-A2H2M50_W4HO	CGAAGTAT	ATCGTACG	2	H2M50_W4HO	H2	M50	W4	HO	16
195BLOCK3-A3H3M50_W4FE	TAGCAGCT	ATCGTACG	2	H3M50_W4FE	H3	M50	W4	FE	16
196BLOCK3-A4H4M50_W4FE	TCTCTATG	ATCGTACG	2	H4M50_W4FE	H4	M50	W4	FE	16
197BLOCK3-A5H5M50_W4FEO	GATCTACG	ATCGTACG	2	H5M50_W4FEO	H5	M50	W4	FEO	16
198BLOCK3-A6H6M50_W4FEO	GTAACGAG	ATCGTACG	2	H6M50_W4FEO	H6	M50	W4	FEO	9
199BLOCK3-A7H1M75_W4HO	ACGTGCGC	ATCGTACG	2	H1M75_W4HO	H1	M75	W4	HO	9
200BLOCK3-A8H2M75_W4HO	ATAGTACC	ATCGTACG	2	H2M75_W4HO	H2	M75	W4	HO	16
201BLOCK3-A9H3M75_W4FE	GCGTATAC	ATCGTACG	2	H3M75_W4FE	H3	M75	W4	FE	16
202BLOCK3-A10H4M75_W4FE	TGCTCGTA	ATCGTACG	2	H4M75_W4FE	H4	M75	W4	FE	16
203BLOCK3-A11H5M75_W4FEO	AACGCTGA	ATCGTACG	2	H5M75_W4FEO	H5	M75	W4	FEO	16
204BLOCK3-A12H6M75_W4FEO	CGTAGCGA	ATCGTACG	2	H6M75_W4FEO	H6	M75	W4	FEO	9
205BLOCK3-B1H1T24_W4HO	CTCGACTT	ACTATCTG	2	H1T24_W4HO	H1	T24	W4	HO	9

BLANK SAMPLE									
207BLOCK3-B3H3T24_W4FE	TAGCAGCT	ACTATCTG	2	H3T24_W4FE	H3	T24	W4	FE	16
BLANK SAMPLE									
209BLOCK3-B5H5T24_W4FEO	GATCTACG	ACTATCTG	2	H5T24_W4FEO	H5	T24	W4	FEO	16
210BLOCK3-B6H6T24_W4FEO	GTAACGAG	ACTATCTG	2	H6T24_W4FEO	H6	T24	W4	FEO	9
211BLOCK3-B7H1T48_W4HO	ACGTGCGC	ACTATCTG	2	H1T48_W4HO	H1	T48	W4	HO	9
212BLOCK3-B8H2T48_W4HO	ATAGTACC	ACTATCTG	2	H2T48_W4HO	H2	T48	W4	HO	16
213BLOCK3-B9H3T48_W4FE	GCGTATAC	ACTATCTG	2	H3T48_W4FE	H3	T48	W4	FE	16
214BLOCK3-B10H4T48_W4FE	TGCTCGTA	ACTATCTG	2	H4T48_W4FE	H4	T48	W4	FE	16
215BLOCK3-B11H5T48_W4FEO	AACGCTGA	ACTATCTG	2	H5T48_W4FEO	H5	T48	W4	FEO	16
216BLOCK3-B12H6T48_W4FEO	CGTAGCGA	ACTATCTG	2	H6T48_W4FEO	H6	T48	W4	FEO	9
217BLOCK3-C1H1T72_W4HO	CTCGACTT	TAGCGAGT	2	H1T72_W4HO	H1	T72	W4	HO	9
218BLOCK3-C2H2T72_W4HO	CGAAGTAT	TAGCGAGT	2	H2T72_W4HO	H2	T72	W4	HO	16
219BLOCK3-C3H3T72_W4FE	TAGCAGCT	TAGCGAGT	2	H3T72_W4FE	H3	T72	W4	FE	16
220BLOCK3-C4H4T72_W4FE	TCTCTATG	TAGCGAGT	2	H4T72_W4FE	H4	T72	W4	FE	16
221BLOCK3-C5H5T72_W4FEO	GATCTACG	TAGCGAGT	2	H5T72_W4FEO	H5	T72	W4	FEO	16
222BLOCK3-C6H6T72_W4FEO	GTAACGAG	TAGCGAGT	2	H6T72_W4FEO	H6	T72	W4	FEO	9
223BLOCK3-C7H1T96_W4HO	ACGTGCGC	TAGCGAGT	2	H1T96_W4HO	H1	T96	W4	HO	9
224BLOCK3-C8H2T96_W4HO	ATAGTACC	TAGCGAGT	2	H2T96_W4HO	H2	T96	W4	HO	16
225BLOCK3-C9H3T96_W4FE	GCGTATAC	TAGCGAGT	2	H3T96_W4FE	H3	T96	W4	FE	16
226BLOCK3-C10H4T96_W4FE	TGCTCGTA	TAGCGAGT	2	H4T96_W4FE	H4	T96	W4	FE	16
227BLOCK3-C11H5T96_W4FEO	AACGCTGA	TAGCGAGT	2	H5T96_W4FEO	H5	T96	W4	FEO	16
228BLOCK3-C12H6T96_W4FEO	CGTAGCGA	TAGCGAGT	2	H6T96_W4FEO	H6	T96	W4	FEO	9
229BLOCK3-D1H1T120_W4HO	CTCGACTT	CTGCGTGT	2	H1T120_W4HO	H1	T120	W4	HO	9
230BLOCK3-D2H2T120_W4HO	CGAAGTAT	CTGCGTGT	2	H2T120_W4HO	H2	T120	W4	HO	16
231BLOCK3-D3H3T120_W4FE	TAGCAGCT	CTGCGTGT	2	H3T120_W4FE	H3	T120	W4	FE	16
232BLOCK3-D4H4T120_W4FE	TCTCTATG	CTGCGTGT	2	H4T120_W4FE	H4	T120	W4	FE	16
233BLOCK3-D5H5T120_W4FEO	GATCTACG	CTGCGTGT	2	H5T120_W4FEO	H5	T120	W4	FEO	16
234BLOCK3-D6H6T120_W4FEO	GTAACGAG	CTGCGTGT	2	H6T120_W4FEO	H6	T120	W4	FEO	9
235BLOCK3-D7H1T144_W4HO	ACGTGCGC	CTGCGTGT	2	H1T144_W4HO	H1	T144	W4	HO	9
236BLOCK3-D8H2T144_W4HO	ATAGTACC	CTGCGTGT	2	H2T144_W4HO	H2	T144	W4	HO	16
237BLOCK3-D9H3T144_W4FE	GCGTATAC	CTGCGTGT	2	H3T144_W4FE	H3	T144	W4	FE	16
238BLOCK3-D10H4T144_W4FE	TGCTCGTA	CTGCGTGT	2	H4T144_W4FE	H4	T144	W4	FE	16
239BLOCK3-D11H5T144_W4FEO	AACGCTGA	CTGCGTGT	2	H5T144_W4FEO	H5	T144	W4	FEO	16
240BLOCK3-D12H6T144_W4FEO	CGTAGCGA	CTGCGTGT	2	H6T144_W4FEO	H6	T144	W4	FEO	9

241BLOCK3-E1H1T168_W4HO	CTCGACTT	TCATCGAG	2	H1T168_W4HO	H1	T168	W4	HO	9
242BLOCK3-E2H2T168_W4HO	CGAAGTAT	TCATCGAG	2	H2T168_W4HO	H2	T168	W4	HO	16
243BLOCK3-E3H3T168_W4FE	TAGCAGCT	TCATCGAG	2	H3T168_W4FE	H3	T168	W4	FE	16
244BLOCK3-E4H4T168_W4FE	TCTCTATG	TCATCGAG	2	H4T168_W4FE	H4	T168	W4	FE	16
245BLOCK3-E5H5T168_W4FEO	GATCTACG	TCATCGAG	2	H5T168_W4FEO	H5	T168	W4	FEO	16
246BLOCK3-E6H6T168_W4FEO	GTAACGAG	TCATCGAG	2	H6T168_W4FEO	H6	T168	W4	FEO	9
247BLOCK3-E7H1M1_W5P	ACGTGCGC	TCATCGAG	2	H1M1_W5P	H1	M1	W5	P3	9
248BLOCK3-E8H2M1_W5P	ATAGTACC	TCATCGAG	2	H2M1_W5P	H2	M1	W5	P3	16
249BLOCK3-E9H3M1_W5P	GCGTATAC	TCATCGAG	2	H3M1_W5P	H3	M1	W5	P3	16
250BLOCK3-E10H4M1_W5P	TGCTCGTA	TCATCGAG	2	H4M1_W5P	H4	M1	W5	P3	16
251BLOCK3-E11H5M1_W5P	AACGCTGA	TCATCGAG	2	H5M1_W5P	H5	M1	W5	P3	16
252BLOCK3-E12H6M1_W5P	CGTAGCGA	TCATCGAG	2	H6M1_W5P	H6	M1	W5	P3	9
253BLOCK3-F1H1M25_W5P	CTCGACTT	CGTGAGTG	2	H1M25_W5P	H1	M25	W5	P3	9
254BLOCK3-F2H2M25_W5P	CGAAGTAT	CGTGAGTG	2	H2M25_W5P	H2	M25	W5	P3	16
255BLOCK3-F3H3M25_W5P	TAGCAGCT	CGTGAGTG	2	H3M25_W5P	H3	M25	W5	P3	16
256BLOCK3-F4H4M25_W5P	TCTCTATG	CGTGAGTG	2	H4M25_W5P	H4	M25	W5	P3	16
257BLOCK3-F5H5M25_W5P	GATCTACG	CGTGAGTG	2	H5M25_W5P	H5	M25	W5	P3	16
258BLOCK3-F6H6M25_W5P	GTAACGAG	CGTGAGTG	2	H6M25_W5P	H6	M25	W5	P3	9
259BLOCK3-F7H1M50_W5P	ACGTGCGC	CGTGAGTG	2	H1M50_W5P	H1	M50	W5	P3	9
260BLOCK3-F8H2M50_W5P	ATAGTACC	CGTGAGTG	2	H2M50_W5P	H2	M50	W5	P3	16
261BLOCK3-F9H3M50_W5P	GCGTATAC	CGTGAGTG	2	H3M50_W5P	H3	M50	W5	P3	16
262BLOCK3-F10H4M50_W5P	TGCTCGTA	CGTGAGTG	2	H4M50_W5P	H4	M50	W5	P3	16
263BLOCK3-F11H5M50_W5P	AACGCTGA	CGTGAGTG	2	H5M50_W5P	H5	M50	W5	P3	16
264BLOCK3-F12H6M50_W5P	CGTAGCGA	CGTGAGTG	2	H6M50_W5P	H6	M50	W5	P3	9
BLANK SAMPLE									
266BLOCK3-G2H2M75_W5P	CGAAGTAT	GGATATCT	2	H2M75_W5P	H2	M75	W5	P3	16
267BLOCK3-G3H3M75_W5P	TAGCAGCT	GGATATCT	2	H3M75_W5P	H3	M75	W5	P3	16
268BLOCK3-G4H4M75_W5P	TCTCTATG	GGATATCT	2	H4M75_W5P	H4	M75	W5	P3	16
269BLOCK3-G5H5M75_W5P	GATCTACG	GGATATCT	2	H5M75_W5P	H5	M75	W5	P3	16
270BLOCK3-G6H6M75_W5P	GTAACGAG	GGATATCT	2	H6M75_W5P	H6	M75	W5	P3	9
271BLOCK3-G7H1T24_W5P	ACGTGCGC	GGATATCT	2	H1T24_W5P	H1	T24	W5	P3	9
272BLOCK3-G8H2T24_W5P	ATAGTACC	GGATATCT	2	H2T24_W5P	H2	T24	W5	P3	16
BLANK SAMPLE									
274BLOCK3-G10H4T24_W5P	TGCTCGTA	GGATATCT	2	H4T24_W5P	H4	T24	W5	P3	16
275BLOCK3-G11H5T24_W5P	AACGCTGA	GGATATCT	2	H5T24_W5P	H5	T24	W5	P3	16

276BLOCK3-G12H6T24_W5P	CGTAGCGA	GGATATCT	2	H6T24_W5P	H6	T24	W5	P3	9
277BLOCK3-H1H1T48_W5P	CTCGACTT	GACACCGT	2	H1T48_W5P	H1	T48	W5	P3	9
278BLOCK3-H2H2T48_W5P	CGAAGTAT	GACACCGT	2	H2T48_W5P	H2	T48	W5	P3	16
279BLOCK3-H3H3T48_W5P	TAGCAGCT	GACACCGT	2	H3T48_W5P	H3	T48	W5	P3	16
280BLOCK3-H4H4T48_W5P	TCTCTATG	GACACCGT	2	H4T48_W5P	H4	T48	W5	P3	16
281BLOCK3-H5H5T48_W5P	GATCTACG	GACACCGT	2	H5T48_W5P	H5	T48	W5	P3	16
282BLOCK3-H6H6T48_W5P	GTAACGAG	GACACCGT	2	H6T48_W5P	H6	T48	W5	P3	9
283BLOCK3-H7H1T72_W5P	ACGTGCGC	GACACCGT	2	H1T72_W5P	H1	T72	W5	P3	9
284BLOCK3-H8H2T72_W5P	ATAGTACC	GACACCGT	2	H2T72_W5P	H2	T72	W5	P3	16
285BLOCK3-H9H3T72_W5P	GCGTATAC	GACACCGT	2	H3T72_W5P	H3	T72	W5	P3	16
286BLOCK3-H10H4T72_W5P	TGCTCGTA	GACACCGT	2	H4T72_W5P	H4	T72	W5	P3	16
287BLOCK3-H11H5T72_W5P	AACGCTGA	GACACCGT	2	H5T72_W5P	H5	T72	W5	P3	16
288BLOCK3-H12H6T72_W5P	CGTAGCGA	GACACCGT	2	H6T72_W5P	H6	T72	W5	P3	9
289BLOCK4-A1H1T96_W5P	CTCGACTT	CTACTATA	2	H1T96_W5P	H1	T96	W5	P3	9
290BLOCK4-A2H2T96_W5P	CGAAGTAT	CTACTATA	2	H2T96_W5P	H2	T96	W5	P3	16
291BLOCK4-A3H3T96_W5P	TAGCAGCT	CTACTATA	2	H3T96_W5P	H3	T96	W5	P3	16
292BLOCK4-A4H4T96_W5P	TCTCTATG	CTACTATA	2	H4T96_W5P	H4	T96	W5	P3	16
293BLOCK4-A5H5T96_W5P	GATCTACG	CTACTATA	2	H5T96_W5P	H5	T96	W5	P3	16
294BLOCK4-A6H6T96_W5P	GTAACGAG	CTACTATA	2	H6T96_W5P	H6	T96	W5	P3	9
295BLOCK4-A7H1T120_W5P	ACGTGCGC	CTACTATA	2	H1T120_W5P	H1	T120	W5	P3	9
296BLOCK4-A8H2T120_W5P	ATAGTACC	CTACTATA	2	H2T120_W5P	H2	T120	W5	P3	16
297BLOCK4-A9H3T120_W5P	GCGTATAC	CTACTATA	2	H3T120_W5P	H3	T120	W5	P3	16
298BLOCK4-A10H4T120_W5P	TGCTCGTA	CTACTATA	2	H4T120_W5P	H4	T120	W5	P3	16
299BLOCK4-A11H5T120_W5P	AACGCTGA	CTACTATA	2	H5T120_W5P	H5	T120	W5	P3	16
300BLOCK4-A12H6T120_W5P	CGTAGCGA	CTACTATA	2	H6T120_W5P	H6	T120	W5	P3	9
301BLOCK4-B1H1T144_W5P	CTCGACTT	CGTTACTA	2	H1T144_W5P	H1	T144	W5	P3	9
302BLOCK4-B2H2T144_W5P	CGAAGTAT	CGTTACTA	2	H2T144_W5P	H2	T144	W5	P3	16
303BLOCK4-B3H3T144_W5P	TAGCAGCT	CGTTACTA	2	H3T144_W5P	H3	T144	W5	P3	16
304BLOCK4-B4H4T144_W5P	TCTCTATG	CGTTACTA	2	H4T144_W5P	H4	T144	W5	P3	16
305BLOCK4-B5H5T144_W5P	GATCTACG	CGTTACTA	2	H5T144_W5P	H5	T144	W5	P3	16
306BLOCK4-B6H6T144_W5P	GTAACGAG	CGTTACTA	2	H6T144_W5P	H6	T144	W5	P3	9
307BLOCK4-B7H1T168_W5P	ACGTGCGC	CGTTACTA	2	H1T168_W5P	H1	T168	W5	P3	9
308BLOCK4-B8H2T168_W5P	ATAGTACC	CGTTACTA	2	H2T168_W5P	H2	T168	W5	P3	16
309BLOCK4-B9H3T168_W5P	GCGTATAC	CGTTACTA	2	H3T168_W5P	H3	T168	W5	P3	16
310BLOCK4-B10H4T168_W5P	TGCTCGTA	CGTTACTA	2	H4T168_W5P	H4	T168	W5	P3	16

311BLOCK4-B11H5T168_W5P	AACGCTGA	CGTTACTA	2	H5T168_W5P	H5	T168	W5	P3	16
312BLOCK4-B12H6T168_W5P	CGTAGCGA	CGTTACTA	2	H6T168_W5P	H6	T168	W5	P3	9
313BLOCK4-C1H1M1_W6FEO	CTCGACTT	AGAGTCAC	2	H1M1_W6FEO	H1	M1	W6	FEO	9
314BLOCK4-C2H2M1_W6FEO	CGAAGTAT	AGAGTCAC	2	H2M1_W6FEO	H2	M1	W6	FEO	16
315BLOCK4-C3H3M1_W6HO	TAGCAGCT	AGAGTCAC	2	H3M1_W6HO	H3	M1	W6	HO	16
316BLOCK4-C4H4M1_W6HO	TCTCTATG	AGAGTCAC	2	H4M1_W6HO	H4	M1	W6	HO	16
317BLOCK4-C5H5M1_W6FE	GATCTACG	AGAGTCAC	2	H5M1_W6FE	H5	M1	W6	FE	16
318BLOCK4-C6H6M1_W6FE	GTAACGAG	AGAGTCAC	2	H6M1_W6FE	H6	M1	W6	FE	9
319BLOCK4-C7H1M25_W6FEO	ACGTGCGC	AGAGTCAC	2	H1M25_W6FEO	H1	M25	W6	FEO	9
320BLOCK4-C8H2M25_W6FEO	ATAGTACC	AGAGTCAC	2	H2M25_W6FEO	H2	M25	W6	FEO	16
321BLOCK4-C9H3M25_W6HO	GCGTATAC	AGAGTCAC	2	H3M25_W6HO	H3	M25	W6	HO	16
322BLOCK4-C10H4M25_W6HO	TGCTCGTA	AGAGTCAC	2	H4M25_W6HO	H4	M25	W6	HO	16
323BLOCK4-C11H5M25_W6FE	AACGCTGA	AGAGTCAC	2	H5M25_W6FE	H5	M25	W6	FE	16
324BLOCK4-C12H6M25_W6FE	CGTAGCGA	AGAGTCAC	2	H6M25_W6FE	H6	M25	W6	FE	9
325BLOCK4-D1H1M50_W6FEO	CTCGACTT	TACGAGAC	2	H1M50_W6FEO	H1	M50	W6	FEO	9
326BLOCK4-D2H2M50_W6FEO	CGAAGTAT	TACGAGAC	2	H2M50_W6FEO	H2	M50	W6	FEO	16
327BLOCK4-D3H3M50_W6HO	TAGCAGCT	TACGAGAC	2	H3M50_W6HO	H3	M50	W6	HO	16
328BLOCK4-D4H4M50_W6HO	TCTCTATG	TACGAGAC	2	H4M50_W6HO	H4	M50	W6	HO	16
329BLOCK4-D5H5M50_W6FE	GATCTACG	TACGAGAC	2	H5M50_W6FE	H5	M50	W6	FE	16
330BLOCK4-D6H6M50_W6FE	GTAACGAG	TACGAGAC	2	H6M50_W6FE	H6	M50	W6	FE	9
331BLOCK4-D7H1M75_W6FEO	ACGTGCGC	TACGAGAC	2	H1M75_W6FEO	H1	M75	W6	FEO	9
332BLOCK4-D8H2M75_W6FEO	ATAGTACC	TACGAGAC	2	H2M75_W6FEO	H2	M75	W6	FEO	16
333BLOCK4-D9H3M75_W6HO	GCGTATAC	TACGAGAC	2	H3M75_W6HO	H3	M75	W6	HO	16
334BLOCK4-D10H4M75_W6HO	TGCTCGTA	TACGAGAC	2	H4M75_W6HO	H4	M75	W6	HO	16
335BLOCK4-D11H4T12_W5P	AACGCTGA	TACGAGAC	2	H4T12_W5P	H4	T12	W5	P3	16
336BLOCK4-D12H6M75_W6FE	CGTAGCGA	TACGAGAC	2	H6M75_W6FE	H6	M75	W6	FE	9
337BLOCK4-E1H1T12_W5P	CTCGACTT	ACGTCTCG	2	H1T12_W5P	H1	T12	W5	P3	9
338BLOCK4-E2H2T12_W5P	CGAAGTAT	ACGTCTCG	2	H2T12_W5P	H2	T12	W5	P3	16
339BLOCK4-E3H3T12_W5P	TAGCAGCT	ACGTCTCG	2	H3T12_W5P	H3	T12	W5	P3	16
340BLOCK4-E4H4T24_W6HO	TCTCTATG	ACGTCTCG	2	H4T24_W6HO	H4	T24	W6	HO	16
341BLOCK4-E5H5T24_W6FE	GATCTACG	ACGTCTCG	2	H5T24_W6FE	H5	T24	W6	FE	16
342BLOCK4-E6H6T12_W5P	GTAACGAG	ACGTCTCG	2	H6T12_W5P	H6	T12	W5	P3	9
343BLOCK4-E7H1T48_W6FEO	ACGTGCGC	ACGTCTCG	2	H1T48_W6FEO	H1	T48	W6	FEO	9
344BLOCK4-E8H2T48_W6FEO	ATAGTACC	ACGTCTCG	2	H2T48_W6FEO	H2	T48	W6	FEO	16
345BLOCK4-E9H3T48_W6HO	GCGTATAC	ACGTCTCG	2	H3T48_W6HO	H3	T48	W6	HO	16

346BLOCK4-E10H4T48_W6HO	TGCTCGTA	ACGTCTCG	2	H4T48_W6HO	H4	T48	W6	HO	16
347BLOCK4-E11H5T48_W6FE	AACGCTGA	ACGTCTCG	2	H5T48_W6FE	H5	T48	W6	FE	16
348BLOCK4-E12H6T48_W6FE	CGTAGCGA	ACGTCTCG	2	H6T48_W6FE	H6	T48	W6	FE	9
349BLOCK4-F1H1T72_W6FEO	CTCGACTT	TCGACGAG	2	H1T72_W6FEO	H1	T72	W6	FEO	9
350BLOCK4-F2H2T72_W6FEO	CGAAGTAT	TCGACGAG	2	H2T72_W6FEO	H2	T72	W6	FEO	16
351BLOCK4-F3H3T72_W6HO	TAGCAGCT	TCGACGAG	2	H3T72_W6HO	H3	T72	W6	HO	16
352BLOCK4-F4H4T72_W6HO	TCTCTATG	TCGACGAG	2	H4T72_W6HO	H4	T72	W6	HO	16
353BLOCK4-F5H5T72_W6FE	GATCTACG	TCGACGAG	2	H5T72_W6FE	H5	T72	W6	FE	16
354BLOCK4-F6H6T72_W6FE	GTAACGAG	TCGACGAG	2	H6T72_W6FE	H6	T72	W6	FE	9
355BLOCK4-F7H1T96_W6FEO	ACGTGCGC	TCGACGAG	2	H1T96_W6FEO	H1	T96	W6	FEO	9
356BLOCK4-F8H2T96_W6FEO	ATAGTACC	TCGACGAG	2	H2T96_W6FEO	H2	T96	W6	FEO	16
357BLOCK4-F9H3T96_W6HO	GCGTATAC	TCGACGAG	2	H3T96_W6HO	H3	T96	W6	HO	16
358BLOCK4-F10H4T96_W6HO	TGCTCGTA	TCGACGAG	2	H4T96_W6HO	H4	T96	W6	HO	16
359BLOCK4-F11H5T96_W6FE	AACGCTGA	TCGACGAG	2	H5T96_W6FE	H5	T96	W6	FE	16
360BLOCK4-F12H6T96_W6FE	CGTAGCGA	TCGACGAG	2	H6T96_W6FE	H6	T96	W6	FE	9
361BLOCK4-G1H1T120_W6FEO	CTCGACTT	GATCGTGT	2	H1T120_W6FEO	H1	T120	W6	FEO	9
362BLOCK4-G2H2T120_W6FEO	CGAAGTAT	GATCGTGT	2	H2T120_W6FEO	H2	T120	W6	FEO	16
363BLOCK4-G3H3T120_W6HO	TAGCAGCT	GATCGTGT	2	H3T120_W6HO	H3	T120	W6	HO	16
364BLOCK4-G4H4T120_W6HO	TCTCTATG	GATCGTGT	2	H4T120_W6HO	H4	T120	W6	HO	16
365BLOCK4-G5H5T120_W6FE	GATCTACG	GATCGTGT	2	H5T120_W6FE	H5	T120	W6	FE	16
366BLOCK4-G6H6T120_W6FE	GTAACGAG	GATCGTGT	2	H6T120_W6FE	H6	T120	W6	FE	9
367BLOCK4-G7H1T144_W6FEO	ACGTGCGC	GATCGTGT	2	H1T144_W6FEO	H1	T144	W6	FEO	9
368BLOCK4-G8H2T144_W6FEO	ATAGTACC	GATCGTGT	2	H2T144_W6FEO	H2	T144	W6	FEO	16
369BLOCK4-G9H3T144_W6HO	GCGTATAC	GATCGTGT	2	H3T144_W6HO	H3	T144	W6	HO	16
370BLOCK4-G10H4T144_W6HO	TGCTCGTA	GATCGTGT	2	H4T144_W6HO	H4	T144	W6	HO	16
371BLOCK4-G11H5T144_W6FE	AACGCTGA	GATCGTGT	2	H5T144_W6FE	H5	T144	W6	FE	16
372BLOCK4-G12H6T144_W6FE	CGTAGCGA	GATCGTGT	2	H6T144_W6FE	H6	T144	W6	FE	9
373BLOCK4-H1H1T168_W6FEO	CTCGACTT	GTCAGATA	2	H1T168_W6FEO	H1	T168	W6	FEO	9
374BLOCK4-H2H2T168_W6FEO	CGAAGTAT	GTCAGATA	2	H2T168_W6FEO	H2	T168	W6	FEO	16
375BLOCK4-H3H3T168_W6HO	TAGCAGCT	GTCAGATA	2	H3T168_W6HO	H3	T168	W6	HO	16
376BLOCK4-H4H4T168_W6HO	TCTCTATG	GTCAGATA	2	H4T168_W6HO	H4	T168	W6	HO	16
377BLOCK4-H5H5T168_W6FE	GATCTACG	GTCAGATA	2	H5T168_W6FE	H5	T168	W6	FE	16
378BLOCK4-H6H6T168_W6FE	GTAACGAG	GTCAGATA	2	H6T168_W6FE	H6	T168	W6	FE	9
379BLOCK4-H7H1T12_W3P	ACGTGCGC	GTCAGATA	2	H1T12_W3P	H1	T12	W3	P2	9
380BLOCK4-H8H2T12_W3P	ATAGTACC	GTCAGATA	2	H2T12_W3P	H2	T12	W3	P2	16

381BLOCK4-H9H3T12_W3P	GCGTATAC	GTCAGATA	2	H3T12_W3P	H3	T12	W3	P2	16
BLANK SAMPLE									
383BLOCK4-H11H5T12_W3P	AACGCTGA	GTCAGATA	2	H5T12_W3P	H5	T12	W3	P2	16
384BLOCK4-H12H6T12_W3P	CGTAGCGA	GTCAGATA	2	H6T12_W3P	H6	T12	W3	P2	9

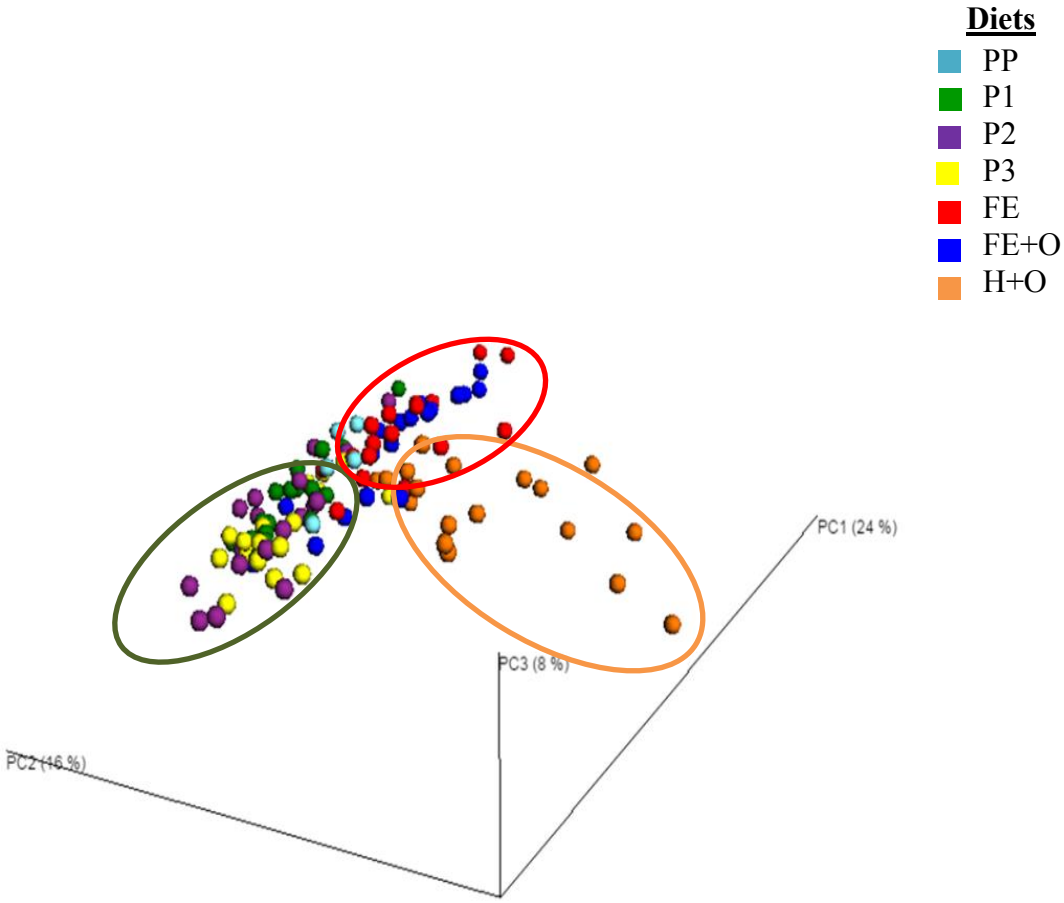
Legend: The linker primer sequence used for the samples was 5'- GTGCCAGCMGCCGCGGTAA-3'. Horses – H1-6; Time points in hours – T0-T168; Time points for % of markers retrieved – M1-M75; Weeks W0-6; Diets – PP (grazing pasture in paddocks); P1, P2 and P3 (cut pasture fed in stables in diet periods 1, 2 and 3, during weeks 1, 3 and 5 respectively); FE (ensiled chopped forage diet); FEO (ensiled chopped forage fed with whole oats); HO (hay fed with whole oats).

F-3. Table S2. Diet-wise comparison of the alpha diversity indices of faecal bacterial genera across the three time points on days 5 (120h), 6 (144h) and 7 (168h) of the study period.

Alpha diversity index	Time points			P value*
	120h	144h	168h	
Diet P1				
Simpson's (diversity)	0.895	0.879	0.895	0.349
Shannon-Weiner (entropy)	2.756	2.612	2.769	0.132
Chao 1 (richness)	60	60	65	0.177
Diet P2				
Simpson's (diversity)	0.887	0.881	0.887	0.667
Shannon-Weiner (entropy)	2.774	2.663	2.768	0.155
Chao 1 (richness)	65	65	65	0.802
Diet P3				
Simpson's (diversity)	0.909	0.915	0.907	0.567
Shannon-Weiner (entropy)	2.912	2.944	2.884	0.155
Chao 1 (richness)	70	67	68	0.989
Diet FE				
Simpson's (diversity)	0.899	0.899	0.893	0.667
Shannon-Weiner (entropy)	2.786	2.798	2.853	0.567
Chao 1 (richness)	61	58	65	0.349
Diet FE+O				
Simpson's (diversity)	0.879	0.895	0.897	0.349
Shannon-Weiner (entropy)	2.725	2.817	2.831	0.081
Chao 1 (richness)	60	62	61	0.349
Diet H+O				
Simpson's (diversity)	0.898	0.886	0.891	0.922
Shannon-Weiner (entropy)	2.783	2.677	2.726	0.922
Chao 1 (richness)	64	61	62	0.959

Legend: *Friedman's test; level of significance was P≤0.016 after Bonferroni correction for multiple comparisons. The median values for alpha diversity indices are presented. Diets are represented as P1, P2, P3 - cut pasture fed during weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage fed with whole oats; and H+O - hay fed with whole oats.

F-4. Figure S1. Principal coordinate analysis of the faecal bacterial communities in samples obtained from horses fed pasture (P1, P2, P3) and three conserved forage diets (FE, FE+O, H+O) on days 5, 6 and 7 of the study period.



Legend: Each dot represents the bacterial community structure in a faecal sample from a horse (H1-6) at a certain time point (T120, T144 and T168). Samples are coloured by diet. The first two principal coordinates PC1 (24%) and PC2 (16%) explained most of the variation. The ellipses around the clusters were drawn manually. Diets were labelled as PP (grazing on pasture in paddocks on day 0), P1, P2 and P3 (cut pasture fed in stables during weeks 1, 3 and 5, respectively), FE (ensiled chopped forage fed in stables), FE+O (ensiled chopped forage fed with oats in stables), and H+O (hay fed with oats in stables).

F-5. Table S3. Relative abundance of the bacterial phyla in the faeces compared across diets.

Phyla	Mean relative abundance						P value*
	P1	P2	P3	FE	FE+O	H+O	
Firmicutes	0.556	0.508	0.467	0.637	0.621	0.489	<0.001*
Bacteroidetes	0.319	0.365	0.376	0.227	0.239	0.214	<0.001*
Spirochaetes	0.032	0.027	0.029	0.052	0.048	0.168	<0.001*
Verrucomicrobia	0.026	0.022	0.043	0.013	0.016	0.032	<0.001*
TM7	0.014	0.007	0.012	0.012	0.015	0.004	<0.001*
Actinobacteria	0.012	0.009	0.015	0.022	0.021	0.027	<0.001*
Unassigned Phyla	0.012	0.012	0.015	0.014	0.011	0.020	0.003*
WPS-2	0.007	0.005	0.005	0.003	0.002	<0.001	<0.001*
Fibrobacteres	0.006	0.022	0.015	0.008	0.012	0.035	<0.001*
Cyanobacteria	0.004	0.010	0.009	0.002	0.003	0.003	<0.001*
Proteobacteria	0.004	0.004	0.004	0.003	0.003	0.002	0.002*
Synergistetes	0.003	0.004	0.005	0.002	0.003	0.001	0.002*
Other Phyla <1%	0.003	0.003	0.003	0.001	0.001	<0.001	<0.001*
Euryarchaeota	0.002	<0.001	<0.001	<0.001	0.001	<0.001	0.274

Legend: *Kruskal-Wallis test; level of significance was P≤0.003 after Bonferroni correction for multiple comparisons. Samples collected on days 5, 6 and 7 of each treatment block were included in the analysis (see methods). Diets are represented as P1, P2, P3 - cut pasture fed during weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage fed with whole oats; and H+O - hay fed with whole oats.

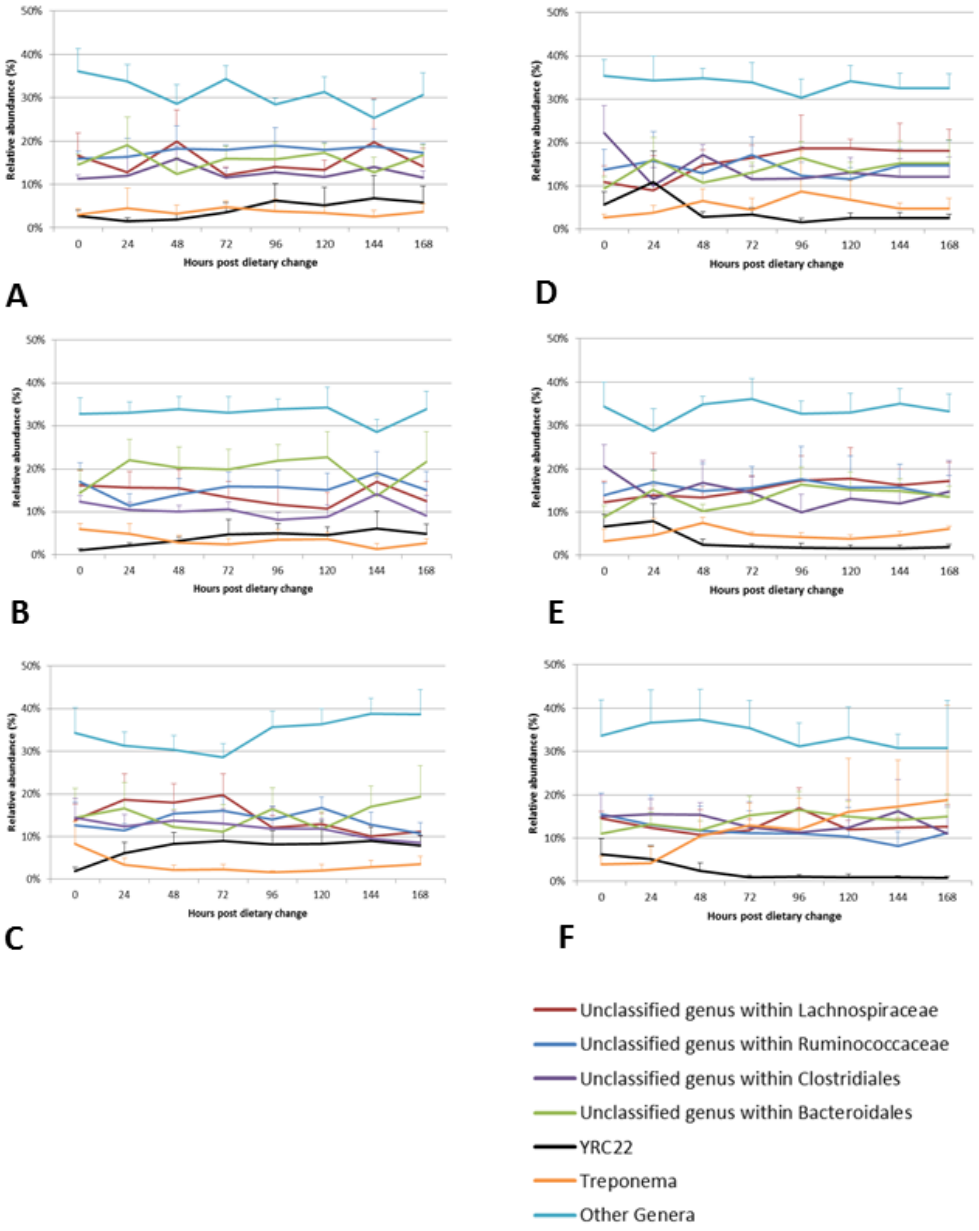
F-6. Table S4. Relative abundance of the bacterial genera in the faeces compared across diets.

	Mean relative abundance						P value*
Genera	**P1**	**P2**	**P3**	**FE**	**FE+O**	**H+O**	
Bacteria>Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>unclassified genus	0.180	0.164	0.135	0.143	0.150	0.100	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.158	0.135	0.114	0.181	0.171	0.125	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>unclassified family>unclassified genus	0.157	0.195	0.16	0.113	0.133	0.134	0.004
Bacteria>Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.125	0.108	0.100	0.145	0.145	0.150	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>YRC22	0.060	0.053	0.084	0.022	0.017	0.009	<0.001*
Bacteria>Spirochaetes>Spirochaetes>Spirochaetales>Spirochaetaceae>Treponema	0.032	0.026	0.029	0.053	0.049	0.169	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>BF311	0.025	0.020	0.018	0.007	0.010	0.011	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>BS11>unclassified genus	0.020	0.020	0.033	0.017	0.017	0.011	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>unclassified genus	0.018	0.014	0.014	0.011	0.013	0.012	0.031
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>CF231	0.018	0.020	0.023	0.031	0.024	0.019	0.032
Bacteria>Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>unclassified genus	0.018	0.014	0.017	0.022	0.022	0.013	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Ruminococcus	0.015	0.028	0.027	0.024	0.024	0.026	0.009
Bacteria>TM7>TM7-3>CW040>F16>unclassified genus	0.014	0.007	0.011	0.012	0.016	0.005	<0.001*
Bacteria>Verrucomicrobia>Verruco-5>WCHB1-41>RFP12>unclassified genus	0.013	0.017	0.030	0.008	0.008	0.010	<0.001*
Bacteria>Verrucomicrobia>Verrucomicrobiae>Verrucomicrobiales>Verrucomicrobiaceae>Akkermansia	0.013	0.006	0.015	0.007	0.010	0.023	0.009
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>Prevotellaceae>Prevotella	0.010	0.013	0.019	0.017	0.013	0.011	0.004
Bacteria>Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>unclassified genus	0.009	0.005	0.009	0.012	0.011	0.010	0.066
Bacteria>WPS-2>unclassified class>unclassified order >unclassified family>unclassified genus	0.008	0.006	0.006	0.003	0.002	0.001	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Clostridium	0.007	0.005	0.008	0.027	0.027	0.011	<0.001*
Bacteria>Fibrobacteres>Fibrobacteria>Fibrobacterales>Fibrobacteraceae>Fibrobacter	0.007	0.023	0.016	0.009	0.013	0.035	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Oscillospira	0.006	0.006	0.004	0.008	0.007	0.006	0.250
Bacteria>Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Phascolarctobacterium	0.006	0.007	0.011	0.021	0.011	0.008	0.009
Bacteria>Firmicutes>Clostridia>Clostridiales>Eubacteriaceae>Pseudoramibacter_Eubacterium	0.006	0.005	0.007	0.003	0.003	0.002	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	0.006	0.004	0.006	0.006	0.007	0.007	0.081
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Coprococcus	0.006	0.006	0.007	0.023	0.021	0.014	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>Porphyromonadaceae>Paludibacter	0.006	0.014	0.014	0.003	0.005	0.002	<0.001*

Bacteria>Firmicutes>Clostridia>Clostridiales>Christensenellaceae>unclassified genus	0.005	0.004	0.005	0.006	0.007	0.005	0.002
Bacteria>Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>Mogibacterium	0.004	0.003	0.006	0.004	0.004	0.002	0.015
Bacteria>Cyanobacteria>4C0d-2>YS2>unclassified family>unclassified genus	0.004	0.011	0.009	0.003	0.003	0.003	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>RF16>unclassified genus	0.004	0.013	0.006	0.002	0.002	0.003	<0.001*
Bacteria>Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>Adlercreutzia	0.004	0.003	0.005	0.009	0.010	0.014	<0.001*
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>S24-7>unclassified genus	0.004	0.002	0.005	0.005	0.006	0.002	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Veillonellaceae>unclassified genus	0.004	0.002	0.003	0.005	0.004	0.002	<0.001*
Bacteria>Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>p-75-a5	0.004	0.002	0.004	0.002	0.002	0.001	<0.001*
Bacteria>Synergistetes>Synergistia>Synergistales>Synergistaceae>unclassified genus	0.003	0.004	0.005	0.002	0.003	0.002	0.003
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.003	0.006	0.004	0.005	0.005	0.004	0.002
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>[Prevotella]	0.003	0.006	0.005	0.007	0.004	0.006	0.108
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	0.003	0.001	0.001	<0.001	<0.001	0.001	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.002	0.005	0.005	0.005	0.005	0.006	0.003
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Pseudobutyrivibrio	0.002	0.005	0.003	0.004	0.002	0.007	<0.001*
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Blautia	0.002	0.002	0.002	0.008	0.007	0.004	<0.001*
Bacteria>Firmicutes>Bacilli>Lactobacillales>Lactobacillaceae>Lactobacillus	0.001	0.002	0.002	<0.001	0.001	0.002	<0.001*
Bacteria>Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>RFN20	0.001	0.002	0.003	0.001	0.001	0.001	<0.001*
Bacteria>Spirochaetes>MVP-15>PL-11B10>unclassified family>unclassified genus	0.001	0.002	0.001	0.001	0.001	0.001	<0.001*
Bacteria>Actinobacteria>Actinobacteria>Actinomycetales>Corynebacteriaceae>Corynebacterium	0.001	0.001	0.001	0.001	0.001	0.001	0.009
Bacteria>Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>[Eubacterium]	0.001	0.001	0.001	0.001	0.001	0.002	0.009
Bacteria>Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>Collinsella	0.001	0.001	0.001	0.002	0.002	0.003	<0.001*
Bacteria>Proteobacteria>Gammaproteobacteria>Pseudomonadales>Moraxellaceae>Acinetobacter	0.001	0.001	0.001	0.001	0.001	0.001	0.165
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>SMB53	0.001	0.001	0.001	<0.001	<0.001	<0.001	<0.001*
Bacteria>Proteobacteria>Alphaproteobacteria>Rhizobiales>Hyphomicrobiaceae>Devosia	<0.001	0.001	0.001	0.001	<0.001	0.001	0.517
Bacteria>Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Megasphaera	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-
Other Genera <1%	0.013	0.014	0.016	0.012	0.014	0.010	0.024
Unassigned Genera	0.012	0.012	0.015	0.014	0.012	0.021	0.004

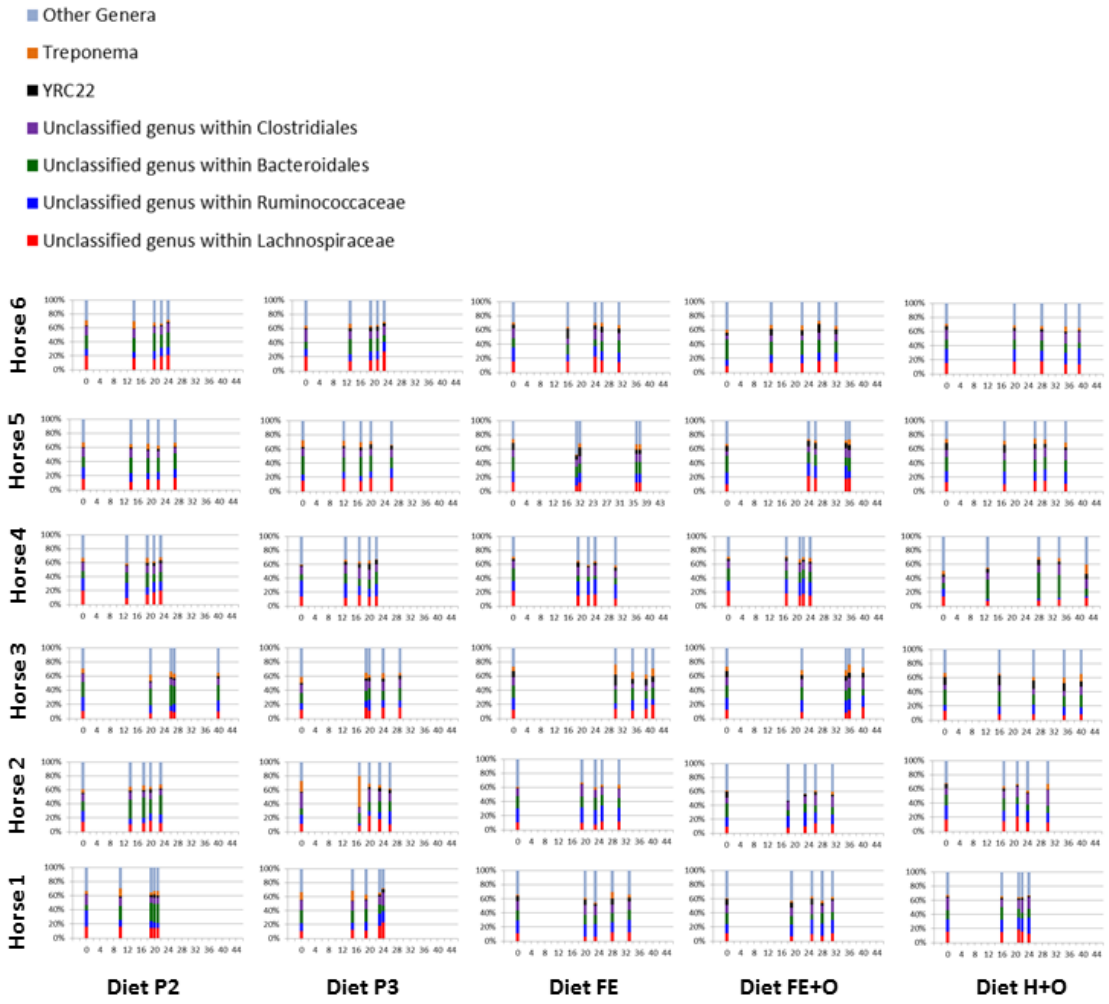
Legend: *Kruskal-Wallis test; level of significance was P≤0.001 after Bonferroni correction for multiple comparisons. Samples collected on days 5, 6 and 7 of each treatment block were included in the analysis (see methods). Diets are represented as P1, P2, P3 - cut pasture fed during weeks 1, 3 and 5, respectively; FE - ensiled chopped forage; FE+O - ensiled chopped forage fed with whole oats; and H+O - hay fed with whole oats.

F-7. Figure S2. Comparison of the relative abundances of the six dominant faecal bacterial genera from 0 to 168 hours following dietary change.



Legend: Mean relative abundances of the six most dominant genera that comprise the faecal bacterial community of horses included in the study are shown in the line graphs. Each panel represents a dietary treatment block. Panel A) Diet P1- cut pasture; Panel B) Diet P2 – cut pasture; Panel C) Diet P3 – cut pasture; Panel D) Diet FE - ensiled chopped forage; Panel E) Diet FE+O - ensiled chopped forage fed with whole oats; Panel F) Diet H+O - hay fed with whole oats. The time points in hours following dietary change are shown on the x-axis of the primary graph in each panel. Error bars represent the standard deviation (only positive shown).

F-8. Figure S3. Comparson of the six most dominant genera with transit time of digesta (T0, M1, M25, M50 and M75) in horses (n=6) across the forage-based diets included in the study.



Legend: Relative abundances of the six most dominant genera and the remaining genera (Other Genera) that comprise the faecal bacterial community of horses included in the study are shown in the stacked bar graphs. The sequence of the stacked bars along the x-axis in each panel is: T0 (time 0 when the diet was changed); M1 (time when the first intestinal marker was retrieved in the faeces); M25 (time when 25% of the intestinal markers were retrieved in the faeces); M50 (time when 50% of the intestinal markers were retrieved in the faeces); and M75 (time when 75% of the intestinal markers were retrieved in the faeces). The time points in hours following dietary change are shown on the x-axis of each graph panel and the relative abundance of the phyla as percentage are shown on the y-axis. The diets are labelled as Diet P2 – cut pasture fed during washout period 2; Diet P3 – cut pasture fed during washout period 3; Diet FE- ensiled chopped forage; Diet FE+O - ensiled chopped forage fed with whole oats; and Diet H+O - hay fed with whole oats.

F-9. Table S5. Relative abundance of faecal bacterial phyla across three time points on day 5 (T120), day 6 (T144) and day 7 (T168) of the study period.

Phyla	Mean relative abundance			P value*
	T120	T144	T168	
Firmicutes	0.574	0.541	0.527	0.343
Bacteroidetes	0.280	0.293	0.301	0.835
Spirochaetes	0.055	0.062	0.064	0.081
Verrucomicrobia	0.019	0.029	0.031	0.253
Fibrobacteres	0.017	0.017	0.017	0.462
Actinobacteria	0.017	0.019	0.018	0.817
Unassigned Phyla	0.013	0.014	0.016	0.644
TM7	0.010	0.011	0.012	0.986
Cyanobacteria	0.005	0.006	0.006	0.317
WPS-2	0.005	0.004	0.004	0.760
Proteobacteria	0.004	0.004	0.004	0.271
Synergistetes	0.004	0.003	0.004	0.199
Other Phyla <1%	0.002	0.003	0.003	0.695
Euryarchaeota	<0.001	0.002	0.002	0.137

Legend: *Friedman's test; level of significance was P≤0.003 after Bonferroni correction for multiple comparisons.

F-10. Table S6. Relative abundance of faecal bacterial genera across three time points on day 5 (T120), day 6 (T144) and day 7 (T168) of the study period.

Genera	Mean relative abundance			P value*
	T120	T144	T168	
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.157	0.143	0.142	0.907
Bacteria>Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>unclassified genus	0.152	0.143	0.141	0.507
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>unclassified family>unclassified genus	0.138	0.152	0.156	0.576
Bacteria>Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.137	0.128	0.121	0.707
Bacteria>Spirochaetes>Spirochaetes>Spirochaetales>Spirochaetaceae>Treponema	0.055	0.061	0.063	0.679
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>YRC22	0.046	0.039	0.038	0.907
Bacteria>Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Ruminococcus	0.027	0.022	0.023	0.105
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>CF231	0.023	0.022	0.023	0.888
Bacteria>Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>unclassified genus	0.017	0.018	0.018	0.635
Bacteria>Fibrobacteres>Fibrobacteria>Fibrobacterales>Fibrobacteraceae>Fibrobacter	0.017	0.017	0.017	0.833
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>BS11>unclassified genus	0.016	0.022	0.021	0.126
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>Bacteroidaceae>BF311	0.014	0.017	0.015	0.268
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>Clostridium	0.014	0.016	0.013	0.904
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>Prevotellaceae>Prevotella	0.014	0.013	0.014	0.720
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>unclassified genus	0.014	0.013	0.013	0.653
Bacteria>Verrucomicrobia>Verruco-5>WCHB1-41>RFP12>unclassified genus	0.012	0.016	0.016	0.119
Bacteria>Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Phascolarctobacterium	0.012	0.009	0.011	0.473
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Coprococcus	0.012	0.013	0.013	0.986
Bacteria>TM7>TM7-3>CW040>F16>unclassified genus	0.010	0.011	0.012	0.307
Bacteria>Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>unclassified genus	0.009	0.010	0.009	0.218
Bacteria>Verrucomicrobia>Verrucomicrobiae>Verrucomicrobiales>Verrucomicrobiaceae>Akkermansia	0.008	0.013	0.016	0.166
Bacteria>Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>Adlercreutzia	0.007	0.007	0.008	0.768

Bacteria>Firmicutes>Clostridia>Clostridiales>Ruminococcaceae>Oscillospira	0.006	0.006	0.006	0.201
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>Porphyromonadaceae>Paludibacter	0.006	0.007	0.008	0.167
Bacteria>Firmicutes>Clostridia>Clostridiales>Christensenellaceae>unclassified genus	0.006	0.006	0.005	0.573
Bacteria>Firmicutes>Clostridia>Clostridiales>unclassified family>unclassified genus	0.005	0.004	0.004	0.048
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>[Paraprevotellaceae]>[Prevotella]	0.005	0.004	0.006	0.703
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Blautia	0.005	0.004	0.004	0.748
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>RF16>unclassified genus	0.005	0.005	0.005	0.461
Bacteria>WPS-2>unclassified class>unclassified order>unclassified family>unclassified genus	0.005	0.004	0.004	0.449
Bacteria>Cyanobacteria>4C0d-2>YS2>unclassified family>unclassified genus	0.005	0.006	0.006	0.925
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>unclassified genus	0.005	0.004	0.004	1.000
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	0.005	0.007	0.006	0.677
Bacteria>Firmicutes>Clostridia>Clostridiales>Eubacteriaceae>Pseudoramibacter_Eubacterium	0.004	0.005	0.004	0.309
Bacteria>Firmicutes>Clostridia>Clostridiales>Lachnospiraceae>Pseudobutyrivibrio	0.004	0.004	0.004	0.922
Bacteria>Bacteroidetes>Bacteroidia>Bacteroidales>S24-7>unclassified genus	0.004	0.004	0.004	0.965
Bacteria>Firmicutes>Clostridia>Clostridiales>[Mogibacteriaceae]>Mogibacterium	0.003	0.005	0.003	0.076
Bacteria>Firmicutes>Clostridia>Clostridiales>Veillonellaceae>unclassified genus	0.003	0.003	0.003	0.508
Bacteria>Synergistetes>Synergistia>Synergistales>Synergistaceae>unclassified genus	0.003	0.003	0.004	0.281
Bacteria>Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>p-75-a5	0.002	0.003	0.003	0.924
Bacteria>Proteobacteria>Alphaproteobacteria>Rhizobiales>Hyphomicrobiaceae>Devosia	<0.001	<0.001	<0.001	-
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>SMB53	<0.001	<0.001	<0.001	0.453
Bacteria>Actinobacteria>Coriobacteriia>Coriobacteriales>Coriobacteriaceae>Collinsella	<0.001	0.002	<0.001	0.602
Bacteria>Firmicutes>Bacilli>Lactobacillales>Lactobacillaceae>Lactobacillus	<0.001	<0.001	<0.001	0.729
Bacteria>Proteobacteria>Gammaproteobacteria>Pseudomonadales>Moraxellaceae>Acinetobacter	<0.001	0.001	0.001	0.541
Bacteria>Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>RFN20	<0.001	<0.001	<0.001	0.958
Bacteria>Actinobacteria>Actinobacteria>Actinomycetales>Corynebacteriaceae>Corynebacterium	<0.001	<0.001	<0.001	0.833
Bacteria>Firmicutes>Clostridia>Clostridiales>Clostridiaceae>unclassified genus	<0.001	<0.001	<0.001	0.989
Bacteria>Firmicutes>Erysipelotrichi>Erysipelotrichales>Erysipelotrichaceae>[Eubacterium]	<0.001	<0.001	<0.001	1.000
Bacteria>Spirochaetes>MVP-15>PL-11B10>unclassified family>unclassified genus	<0.001	<0.001	<0.001	0.921

Bacteria>Firmicutes>Clostridia>Clostridiales>Veillonellaceae>Megasphaera	<0.001	<0.001	<0.001	-
Other Genera <1%	0.012	0.013	0.014	0.115
Unassigned Genera	0.013	0.014	0.016	0.232

Legend: *Friedman's test; level of significance was P≤0.001 after Bonferroni correction for multiple comparisons.

CHAPTER 9

GENERAL DISCUSSION AND FUTURE DIRECTIONS

CHAPTER 9: GENERAL DISCUSSION AND FUTURE DIRECTIONS

9.1 General discussion

The New Zealand Pony Clubs Association is the largest organised group of leisure and competition horse owners within the general horse population outside the racing industry (Anonymous, 2012). As communication between the regional groups of Pony Club horse owners is dominated via social media, an online survey tool was used to distribute and collect information on the feeding, management, and health of horses and ponies in New Zealand (Chapter 3). The survey identified that the majority of horses and ponies were managed continuously on pasture throughout the year, and there was no seasonal variation in the hours allowed for grazing. This may be due to the availability of a perennial pasture cover in New Zealand, which makes it an economically viable option and a major source of dietary nutrition for feeding domestic horses (Hoskin and Gee, 2004). Management of horses on pasture in New Zealand is similar to that in Australia, but is in contrast to North America and Great Britain, where seasonal changes constrain horse owners to manage their animals intensively for most parts of the year (Huntington and Jenkinson, 1998, Christie *et al.*, 2004, Hotchkiss *et al.*, 2007). The main nutrition-related digestive or gastrointestinal (GI) disturbances reported in the Pony Club animals managed on pasture were obesity, laminitis, colic and grass staggers. Although lower than the average reported in other countries, 22% of the horses and ponies kept on pasture were identified to be obese (BCS≥7) by their owners, and this aspect was then further investigated.

A seasonal change in herbage mass and nutrient composition occurs in New Zealand pastures, which was expected to affect the dry matter intake (DMI) and body condition of horses grazing on pasture throughout the year (Litherland *et al.*, 2002, Hirst, 2011, Giles *et al.*, 2014). A subset of the Pony Club horse population within a local region were surveyed prospectively (Chapter 4), providing an opportunity to collect accurate and repeated physical measurements of body morphology of horses at events organised during two seasons (spring and autumn), and to provide evidence of any consistent differences in body condition within the same animals. The study allowed the collection of objective data on adiposity in the animals that was measured by a single assessor, which was a more reliable method of

estimation of body condition when compared to subjective scores reported by the horse owners previously (Chapter 3; (Carter *et al.*, 2009). The results indicated an overall tendency for owners to underestimate obesity in their horses, especially ponies. This potential for underestimation of body condition by owners suggests that the percentage of obese horses and ponies identified in the first survey may have been underestimated as well. Therefore, a potentially higher percentage of obese animals may exist in the New Zealand Pony Club horse population, which supports the global concerns of equine obesity in leisure horses and ponies (Sillence, 2012).

The results of the second survey also showed that most Pony Club horses were healthy when managed on pasture, and maintained body weight from spring to autumn. Since equids can derive most of their energy requirements from microbial fermentation of forage in the hindgut, the results of this work raised some important questions about the relationship between dietary management and the microbiota populations in the hindgut. We hypothesised that the altered DMI, due to an increase or decrease in pasture availability and feed consumption, would affect the transit time of digesta and the population of microbiota in the hindgut, and this effect may have contributed to the maintenance of body weight across seasons by the Pony Club horses, as observed previously (Chapter 4). Furthermore, the inconsistent supplementation of horses kept on pasture with a variety of feeds (Chapter 3) highlighted the possibility for the occurrence of digestive disturbances due to abrupt changes in the availability of dietary substrate for fermentation in the hindgut. Therefore, a series of experimental studies further investigated the effects of common dietary management practices on the faecal microbiota of horses in New Zealand.

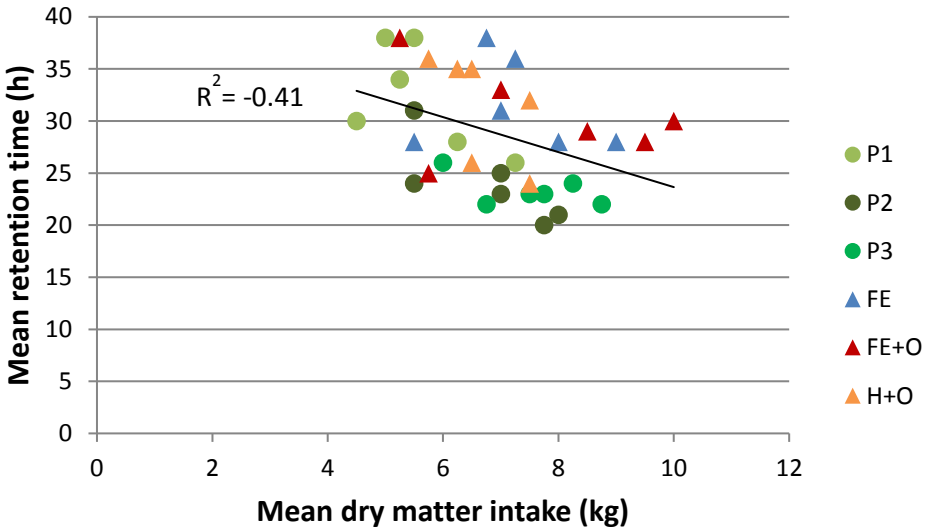
In the first of these studies (Chapter 5), a diet-specific microbiota profile was identified in horses that were maintained on two different forage diets (pasture versus an ensiled chopped forage-based diet). Previous studies have shown differences in the faecal microbiota profiles of horses fed high-fibre, high-fat or high-starch diets (Dougal *et al.*, 2014). The two types of forage diets selected in our study were less extreme nutritionally than those used by Dougal and co-workers, yet significant differences in the faecal microbiota profiles were still identified. These findings indicated that the population of microbiota were strongly influenced by the dietary substrates available in the hindgut, and that even mild changes in the source of dietary fibre could significantly influence the faecal microbiota profile. An abrupt dietary change in the study from an ensiled chopped forage-based diet to pasture resulted in a rapid shift in the microbiota profile, with significant differences observed by the fourth day.

However, there were fluctuations in the faecal microbiota profiles of the horses which continued to graze on pasture over the subsequent 3-week study period. These fluctuations in microbiota populations and the association with potential changes in pasture composition were investigated further using a cohort of horses that were managed on pasture and followed through a year (Chapter 6). The study showed that the pasture composition significantly differed across seasons, which was correlated with seasonal climatic changes (such as ambient temperature and rainfall), and resulted in significant differences in the diversity and community structure of the faecal microbiota. Therefore, it is likely that the fluctuations in the microbiota populations previously observed (Chapter 5), were also due to continuous changes in the pasture composition. This suggests that continuous fluctuations in the faecal microbiota population, within a normal range, can be expected, depending on the dietary substrate in the hindgut.

The information on diet and management obtained from this series of studies was then used to design a final randomised controlled feeding trial (Chapters 7 and 8). In the previous work (Chapter 5), the management of the horses (i.e. feeding ensiled chopped forage-based diet in stables versus grazing on pasture in paddocks) and the type of forage diets (e.g. in physical form, moisture content, nutrient composition, and perhaps palatability) was different, and this may have contributed to the shift in microbiota profile following abrupt dietary change. In this previous work, the degree of association between microbiota profiles and diet or management could not be determined. Furthermore, it was difficult to measure the feed intake of individual horses grazing on pasture, because the horses were set stocked and allowed *ad libitum* access to grazing in paddocks as a herd (Chapters 5 and 6). Therefore, the final controlled experiment was designed to investigate the effects of abrupt dietary transition on the microbiota profile when the management was kept consistent through the study period. The variation observed between horses and the response time for a change in microbiota profile following dietary transition was also thought to be associated with the mean retention time of digesta, and these aspects were investigated further (Chapters 7 and 8).

The first part of the final feeding trial investigated the mean retention time (MRT) of digesta in the GI tract (Chapter 7). The analysis identified significant differences in the MRT of four forage-based diets. The diets with a high moisture content (cut pasture) were consumed more quickly, which resulted in a higher DMI and a shorter mean retention time in the GI tract, when compared to the conserved forage diets (ensiled chopped forage and hay) that contained high dry matter (Figure 9.1).

Figure 9.1. Association between mean dry mater intake and mean retention time in horses fed cut pasture and three conserved forage diets.



Legend: The pasture diets are labelled as P1, P2 and P3, for cut pasture fed in weeks 1, 3 and 5, respectively. The conserved forage diets are labelled as FE (ensiled chopped forage), FE+O (ensiled chopped forage with whole oats) and H+O (hay fed with whole oats). R^2 is the coefficient of correlation between mean dry matter intake (kg) and mean retention time of digesta in the gastrointestinal tract (h).

Despite some differences in the rate of feed intake and MRT of digesta between individual horses, all horses maintained body weight and condition during the feeding trial (Chapter 7). This finding suggests that the capability of ponies to maintain body condition despite the seasonal variation in pasture composition reported earlier (Chapter 4), may also have been associated with the rate of feed intake and the MRT for fermentation of digesta in the hindgut. This negative correlation between DMI and MRT (Figure 9.1) supports our hypothesis that, under restricted grazing conditions for management of obese ponies (Argo *et al.*, 2012), or when the DMI decreases due to seasonal availability of pasture (Chapter 4), the MRT of digesta increases, thereby providing more time for microbial fermentation in the hindgut to serve energy requirements. This compensatory digestive strategy has previously been described in donkeys that are capable of utilising poor quality forage (Pearson and Merritt, 1991, Pearson *et al.*, 2001). An important implication of this association may be applicable when providing restricted grazing for obese ponies under a weight-loss

management program, wherein the potential differences in digesta transit times due to differences in feed intake levels should be considered to effectively maintain the targeted negative energy balance.

The nutrient composition of New Zealand pasture is influenced by climatic conditions such as ambient temperature and rainfall (Chapter 6), and the microbiota profile is specific to the type of conserved forage that is prepared from pasture (Chapters 5, 6 and 8). A more consistent microbiota profile was observed in horses that were fed the conserved forage diets containing ensiled chopped forage, with or without supplementation of whole oats, when compared to the cut pasture and hay diets (Chapter 8). This ensiled chopped forage also had a more consistent nutrient profile when compared to pasture and hay (Chapter 7). These findings indicate that the type of forage (e.g. pasture versus conserved forage, or chopped versus long-stem forage), the time of harvest (e.g. fixed time during the year at a mature growth stage for harvesting the ensiled chopped forage versus variable time and growth stages for harvesting hay), and the method of preparation of the conserved forages (e.g. controlled fermentation in polyethylene packaging versus sun drying and open barn storage), appear to be important factors that may affect the nutrient composition of forage and in turn the microbiota populations in the hindgut. Therefore, these dietary factors need to be considered when developing feeding strategies for horses that are predisposed to disturbances in the microbial populations of the hindgut. Based on the results presented (Chapters 7 and 8), it may be suggested that feeding a more consistent diet may support a more stable hindgut microbiota.

The findings in this thesis support the hypothesis that the faecal microbiota of horses is strongly diet-specific and resilient following dietary change, providing there is a high proportion of forage in the diet. These findings document further evidence of the association between dietary management and the faecal microbiota of horses, as previously described in some studies on the equine faecal or hindgut microbiota (O' Donnell *et al.*, 2013, Dougal *et al.*, 2014, Schoster *et al.*, 2015). Metagenomics of the 16S rRNA gene used in this thesis enabled quantification of the diversity and community structure of bacterial genera, and the results unveiled new information that contributed to our growing knowledge on the equine faecal microbiome.

9.2 Implications for the equine industry

Owners appear to underestimate obesity, especially in pony breeds. This is concerning given the emerging global issue of equine obesity in the leisure horse population and the increased risk for certain diseases such as laminitis. Obesity among leisure horses in New Zealand needs to be addressed and awareness on the assessment of adiposity and management of an ideal body condition should be promoted among owners of pony breeds. In order to improve the management of obese ponies, the findings of this thesis have been disseminated to Pony Club horse owners in New Zealand via the New Zealand Pony Club newsletter. Awareness on monitoring body condition of individual horses, and training to correctly use morphometric measurements for assessing adiposity in horses, and especially in ponies, is recommended for owners of pony breeds.

The association between digesta transit time and the microbiota population should also be considered when restricting the diet of obese ponies, intensively managed competition horses, and during abrupt dietary transition in animals around competition regimens. Abrupt dietary changes are a risk factor for the development of colic, a condition that is also associated with perturbation of the hindgut microbiota (Venable, 2011, Costa *et al.*, 2012). Furthermore, the variation in digesta transit time and the microbiota populations observed between individual horses, places further importance on the factors associated with increased predisposition of some horses to GI disturbances compared to others that are less susceptible. This implies that intensive management (e.g. forage restrictions and abrupt dietary changes), individual horse feeding behaviours (e.g. feed preference and voluntary intake rates), and nutritional requirements (e.g. weight-loss, maintenance and light-, moderate-, or heavy-work) need to be considered when formatting dietary regimens.

This thesis identified an association between dietary management and faecal microbiota populations, and showed significant changes in the diversity and relative abundance of the faecal microbiota in response to abrupt dietary transition in clinically normal horses. Seasonal changes and subtle daily changes in the nutrient composition of the diet influence the microbiota population continuously, but the population dynamics of the faecal microbiota appears to be resilient. Therefore, the microbiota appears capable of continuously adapting to the dietary intake of the horse in order to optimise feed conversion. The data on feed intake, digesta transit time, and microbiota changes presented in this thesis should be considered for intensively managed horses and those prone to GI disturbances, particularly

following abrupt dietary transitions, even when forage is the major component of the diet. Feeding large proportions of forage, and a diet that has a consistent nutrient profile are recommended for maintaining a more stable microbiota population.

9.3 Future research directions

There are several questions that arise from the work presented in this thesis, which future studies should seek to answer. The variation in the microbiota profiles observed between horses was attributed to individual horse factors such as the DMI of feed and the MRT of digesta in the GI tract. However, the fundamental aspects of the feeding behaviour that drives the transit time of digesta is an important area that should be addressed to investigate the trade-off between intake and MRT an individual horse makes to optimise digestion to meet its energy requirements for maintenance of body weight and performance. Further investigations should focus on the behaviour of horses, to understand the effects of feed preferences, palatability, rate of feed consumption, and voluntary DMI on the MRT of digesta in the GI tract in different groups of horses (such as racing, competition and leisure horses and ponies). The relationship between dietary substrate concentration and the transit time of digesta in the GI tract, and their combined effects on the faecal microbiota population should also be explored.

The diversity of faecal microbiota may have been influenced by the management of horses during the study periods (e.g. grazing pasture versus feeding on cut pasture in stables), which could be associated with the activity levels of the horses (free exercise or lack thereof). The activity levels of the horse may influence the motility of the GI tract and transit time of digesta. Therefore, further observational work should be directed towards understanding the effects of routine dietary management practices on the motility of the GI tract and the diversity and population dynamics of the faecal microbiota, especially around performance during competition and training.

The GI microbiota has been associated with the development and progression of obesity (Graham *et al.*, 2015). Notable perturbations in the bacterial communities in obese individuals have been reported, and research is on-going to distinguish between the obesogenic mechanisms attributable to excessive energy in the diet, and those that may be associated with the gut microbiota. There is some evidence from animal and human studies that the microbiota in the obese individual harvests energy more effectively and may manipulate host gene

function leading to increased adiposity, aggravation of inflammatory mechanisms, metabolic endotoxaemia, and metabolic dysfunction (Geor, 2008, Johnson *et al.*, 2009, Respondek *et al.*, 2011). Unique microbiota profiles have been identified in diseased states such as equine metabolic syndrome, colitis and laminitis (Costa *et al.*, 2012, Moreau *et al.*, 2014, Elzinga *et al.*, 2016). Therefore, it is likely that harbouring an obesogenic or chronically perturbed gut microbiota may be linked to the increased susceptibility of some animals to disease conditions (Longland and Byrd, 2006), and this aspect requires further investigation.

This thesis examined the population dynamics of the faecal microbiota in normal horses and provides a baseline from which future studies can be designed. As mentioned earlier, there is emerging evidence on the characteristics of the faecal and hindgut microbiota of horses in diseased states (such as obesity, metabolic syndrome, laminitis and colic). The important findings on the composition and resilience of the bacterial community reported in this thesis could be directed towards developing clinical nutrition strategies to manipulate the gut microbiota in diseased states, in order to return the perturbed microbiota profile back to normalcy. These strategies could include the development and administration of prebiotics, probiotics, direct fed microbials, bacteriotherapy and faecal microbial transplant (Mullen *et al.*, 2016). Further research on the usefulness and efficacy of microbial therapy in convalescent horses requires attention.

Since 2012, many equine microbiota studies have uncovered information on the structure of the bacterial community in the equine hindgut and faeces. The results of the studies presented in this thesis provide key data that has contributed new information to the current knowledge on the equine faecal microbiome. The use of high-throughput sequencing technology expanded our capability to capture the microbial diversity in the faeces, and enabled comparisons with recent studies on the equine faecal microbiota in the past four years. However, there is a dearth of information on other microbiota, such as archaea, protozoa and fungi that also have important functions in the digestive health of the horse. Our investigations were limited to the population dynamics of the faecal bacterial community of forage-fed horses, but many other aspects around the complexity of the microbiota population (such as the diversity and structure of other microbial communities) and their function in the equine hindgut ecosystem are still unclear, and require further investigation. A greater sequencing depth (~600-700 bp) is required for species identification of these microbes, the majority of which are anaerobic and have not yet been identified. Furthermore, the functional significance of members of the microbiota communities and their metabolites, their role in the fermentation

process, and the perturbations that occur in proximal regions of the hindgut in association with disease, are still poorly understood and require further investigation.

Future studies should consider alternate methodologies using high-throughput sequencing with greater depth, shotgun sequencing, functional genomics, metabolomics, and a complex systems approach to understand the function and stability of this microbiome, and to link the interactions of the microbiota to functional pathways within this complex ecosystem. The need for conducting randomised controlled experiments, with larger sample sizes, is emphasised to minimise variation between individual animals, diets, management and other environmental factors. There is also a need to standardise the laboratory, sequencing and bioinformatics methodologies, to minimise variation in the diversity and community structure of the microbiota due to methodology biases.

The results of this thesis lay the foundation for future studies to investigate if dietary strategies can be developed and successfully implemented to reduce GI disturbances in horses and ponies.

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APPENDIX G

(PUBLICATIONS)

APPENDIX G: PUBLICATIONS

G-1. List of peer-reviewed research articles.

G-2. Pdf copies of published articles.

G-3. News article by Horsetalk (November 2014).

G-4. News article by Kentucky Equine Research (December 2014).

G-5. News article by Kentucky Equine Research (April 2015).

G-6. New Zealand Pony Clubs Association, I-Coach Newsletter (June 2015).

G-7. List of research presentations.

G-8. Statements of contribution to doctoral thesis containing publications.

G-1. List of peer-reviewed research articles.

Fernandes, K. A., Rogers, C. W., Gee, E. K., Bolwell, C. F. and Thomas, D. G. (2014). A cross-sectional survey of rider and horse demographics, and the feeding, health and management of Pony Club horses in New Zealand. *Proceedings of the New Zealand Society of Animal Production.* Napier, 74: 11-16.

Fernandes, K. A., Kittelmann, S., Rogers, C. W., Gee, E. K., Bolwell, C. F., Bermingham, E. N. and Thomas, D. G. (2014). Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. *PLoS ONE,* 9: e112846.

Fernandes, K. A., Rogers, C. W., Gee, E. K., Bolwell, C. F. and Thomas, D. G. (2015). Body condition and morphometric measures of adiposity in a cohort of Pony Club horses and ponies in New Zealand. *Proceedings of the New Zealand Society of Animal Production.* Dunedin, 75:195-199.

Fernandes, K. A., Gee, E. K., Rogers, C. W., Kittelmann, S., Biggs, P. J., Bermingham, E. N., Bolwell, C. F. and Thomas, D. G. (2016). Seasonal variation in the faecal microbiota of mature adult horses maintained on pasture in New Zealand. *PLoS ONE,* Submitted.

Fernandes, K. A., Rogers, C. W., Gee, E. K., Fitch, G., Bolwell, C. F., Kittelmann, S., Bermingham, E. N. and Thomas, D. G. (2016). Comparison of gastrointestinal transit times in stabled Thoroughbred horses during abrupt dietary transition between freshly cut pasture and three conserved forage-based diets. *BMC Veterinary Research,* Submitted.

Fernandes, K.A., Rogers, C.W., Gee, E.K., Kittelmann, S., Bolwell, C.F., Bermingham, E.N., Biggs, P.J. and Thomas, D.G. (2016). Resilience in the population dynamics of faecal microbiota in stabled Thoroughbred horses following abrupt dietary transition between freshly cut pasture and three forage-based diets. *PLoS ONE,* Submitted.

CHAPTER 5

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Faecal Microbiota of Forage-Fed Horses in New Zealand and the Population Dynamics of Microbial Communities following Dietary Change

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Abstract

The effects of abrupt dietary transition on the faecal microbiota of forage-fed horses over a 3-week period were investigated. Yearling Thoroughbred fillies reared as a cohort were exclusively fed on either an ensiled conserved forage-grain diet ("Group A"; n = 6) or pasture ("Group B"; n = 6) for three weeks prior to the study. After the Day 0 faecal samples were collected, horses of Group A were abruptly transitioned to pasture. Both groups continued to graze similar pasture for three weeks, with faecal samples collected at 4-day intervals. DNA was isolated from the faeces and microbial 16S and 18S rRNA gene amplicons were generated and analysed by pyrosequencing. The faecal bacterial communities of both groups of horses were highly diverse (Simpson's index of diversity >0.8), with differences between the two groups on Day 0 ($P<0.017$ adjusted for multiple comparisons). There were differences between Groups A and B in the relative abundances of four genera, BF311 (family Bacteroidaceae; $P = 0.003$), CF231 (family Paraprevotellaceae; $P = 0.004$), and currently unclassified members within the order Clostridiales ($P = 0.003$) and within the family Lachnospiraceae ($P = 0.006$). The bacterial community of Group A horses became similar to Group B within four days of feeding on pasture, whereas the structure of the archaeal community remained constant pre- and post-dietary change. The community structure of the faecal microbiota (bacteria, archaea and ciliate protozoa) of pasture-fed horses was also identified. The initial differences observed appeared to be linked to recent dietary history, with the bacterial community of the forage-fed horses responding rapidly to abrupt dietary change.

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Introduction

The horse is a cursorial grazer with the ability to efficiently utilise high-fibre grass and other forages [1,2]. The majority of ingested plant fibre is comprised of structural carbohydrates such as cellulose, hemicellulose, and lignin, which cannot be digested by host enzymes in the foregut. As a result, the undigested plant material reaches the hindgut where breakdown of cellulose and hemicellulose occurs through the process of microbial fermentation, generating energy-yielding products such as volatile fatty acids (VFAs) [3,4]. It is estimated that forage-fed horses may obtain 50–70% of their energy requirements from VFAs [5].

Several species of microbes including bacteria, archaea and eukarya (protozoa and fungi) inhabit the equine gastrointestinal tract [6,7]. However, the bacterial community, which represents the major proportion of the hindgut microbiota, has been the focus of much of the published literature and has predominantly been investigated using culture-based techniques [8,9,10]. Other molecular techniques independent of culture, such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), fluorescent in-situ hybridization (FISH) and terminal restriction fragment length polymorphism (TRFLP), have also been used [8,11,12]. More recently, a few investigations have used metagenomics involving high-throughput next generation sequencing (pyrosequencing of 16S and 18S rRNA gene amplicons) to explore the equine hindgut microbiome (eHGM) *in vivo* [13,14,15]. However, there are still gaps in the current knowledge on the composition and the function of the microbial communities that inhabit the equine hindgut. This may be due to the difficulty in culturing anaerobic microorganisms and comparing results from studies that have used different experimental designs, sequencing methodologies and phylogenetic and statistical data analyses [8].

Because of the need to fistulate the horse to obtain hindgut samples, the majority of the work on the eHGM has focused on the use of material obtained from faecal samples [8]. From the limited comparative work conducted on the bacterial community of the equine hindgut and faeces, there appears to be good agreement between the microbiomes of the colon and faeces [15,16].

The population of hindgut microbiota is sensitive to changes occurring in the gastrointestinal environment, with variations in the bacterial community structure reported in response to the composition of a diet, passage rate of digesta, and level of pre-caecal starch digestion [17,18]. Abrupt dietary changes, particularly the availability of fermentable starch and water-soluble carbohydrates, can perturb the populations of microbes in the hindgut and can lead to digestive and metabolic disorders such as hindgut acidosis, colic and laminitis [6,19,20]. Forage-only diets promote greater microbial stability, evident by the lower microbial counts and relative abundances of specific lactic acid producing bacteria [11]. However, the effects of changes in forage-based diets on the bacterial population in the hindgut or faeces may be dependent on the type of horse (breed), composition of the diet and management practices [12,21,22].

Culture-based techniques have identified that faecal colony forming units of *Streptococcus* spp. and *Lactobacillus* spp. increase significantly from pasture baseline values within six days of an acute dietary transition to a grain-based diet, with numbers subsequently decreasing when the horses were returned back to grazing on pasture [23]. Similarly, an abrupt change from feeding 100% hay diets to a combination of hay and concentrates was reported to produce changes in the hindgut bacterial populations within 5 hours (caecum) and 29 hours (colon), with greater changes occurring in the colon [24]. More recently, an *in vitro* study suggested that bacterial communities isolated from horse faeces responded to a carbohydrate substrate within 12 hours, with significant changes in relative abundances of bacteria over a 48-hour period [25]. The above findings indicate that changes in the bacterial community occur rapidly within the hindgut, and may be evident in the faeces within a few days, depending on the type of diet and the transit time through the intestinal tract.

In New Zealand, equine pastures are predominantly comprised of a perennial ryegrass and white clover mix, with lesser quantities of other grasses and legumes, and many horses are kept or reared on pasture all year round [26,27,28]. The composition of microbiota in the faeces of New Zealand pasture-fed horses, or the effects of an abrupt dietary change from forage-grain diets to pasture on the hindgut or faecal microbiota, are currently unknown. The aims of the current study were to: 1) describe and compare the faecal microbiota of horses grazing on pasture to those fed exclusively on an ensiled conserved forage-grain diet in loose-boxes; and 2) investigate the changes in the relative abundance of faecal microbiota due to abrupt dietary change from an ensiled conserved forage-grain diet to pasture over a period of three weeks.

Materials and Methods

Ethics statement

The use of animals, including welfare, husbandry, experimental procedures, and collection of the faecal samples for this study, was approved by the Massey University Animal Ethics Committee (MUAEC), Massey University, Palmerston North, New Zealand (Protocol number 12–51).

Experimental design and sample collection

Twelve yearling Thoroughbred fillies (mean age ± standard deviation [SD], 396±22 days) born and reared as a cohort on a commercial Thoroughbred stud farm (Palmerston North, Manawatu, New Zealand) were enrolled in the study during the spring of 2012 (November). Ten of the yearlings were sired by the same stallion. The general health and history of feeding management were recorded, and included daily observations by the stud master for any signs of illness or disease, weekly measurements of height, weight and body condition scores, anthelminthic treatments, and veterinary check-ups. The yearlings were in good health and had a median body condition score of six (Interquartile range [IQR] 5–6) on a 9-point scale [29], and a mean height and weight of 146.3±2.8 cm and 348.3±21.7 kg, respectively.

The 12 yearling horses were randomly divided into two treatment groups. For 21 days prior to Day 0, horses in Group A (n = 6) were kept in loose-boxes (4×4 m) lined with rubber matting, and were fed exclusively on a commercial ensiled conserved forage-grain-based ration (Diet F - FiberSure, Fiber Fresh Feeds Ltd., Reporoa, New Zealand), as part of a voluntary feed intake and digestibility study [30]. Diet F comprised of ensiled chaffed lucerne (*Medicago sativa*; alfalfa grass; 65%), cracked maize (*Zea mays*) grain (35%) and a vitamin and mineral premix with molasses (5%); the nutrient analysis of the diet is given in Table S1. The loose-boxes were arranged in a single row, the lower half of the internal walls were made of wood and the upper half made of wire mesh, allowing visual contact between all horses in the loose-boxes and the adjacent yard. The horses were turned-out for exercise in pairs, in a compact-earth yard adjacent to the loose-boxes, for 30 minutes twice a day [30]. Horses in Group B were kept in a paddock and were provided *ad libitum* pasture (Diet P; a standard New Zealand ryegrass-clover pasture comprised of ~80–95% perennial ryegrass (*Lolium perenne*) and ~5–20% white clover (*Trifolium repens*) [27]) for 21 days prior to Day 0. After the Day 0 faecal samples were collected, horses in Group A were abruptly transitioned to feeding on pasture (Diet P). Both groups of horses continued to graze on pasture for the next three weeks (21 days), during which the horses were kept in separate 1.5–2.0 hectare paddocks on the same property, containing pasture of similar herbage mass (pasture cover of 1600–2000 kg DM/ha/year) and nutrient content (Table S1).

Faecal samples were collected from all yearlings between 0900 and 1200 hours on Day 0, and subsequently at 4-day intervals over a period of 21 days (giving a total of 72 samples). The samples were collected within two minutes of defecation, using a forceps to collect representative faecal samples with minimal environmental contamination. These were immediately transferred into 3 ml polyethylene cryovials (Ray Lab Ltd., Auckland, New Zealand), and snap frozen in liquid nitrogen. The faecal samples were stored in a portable canister containing liquid nitrogen and transferred to a −80°C freezer within four hours of collection and stored until laboratory analysis.

DNA extraction, PCR amplification of target genes and pyrosequencing

Nucleic acids were extracted from 100 mg of faeces by disrupting the cells by a combined bead-beating and phenol-chloroform-isoamyl alcohol (25:24:1; vol:vol:vol) treatment and subsequent precipitation of proteins with chloroform [31]. DNA was precipitated from the aqueous phase with two volumes of 30% (wt:vol) polyethylene glycol, washed with 70% (vol:vol) ice-cold ethanol, dried and eluted in 50 µl of elution buffer (EB; 10 mM Tris, pH 8.5 with HCl). Extracted DNA was quantified using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and normalized to 40 ng/µl.

Polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA genes (V1–V3 regions), archaeal 16S rRNA genes (V6–V8 regions), and ciliate protozoal 18S rRNA genes (V5–V8 regions) were carried out as described previously, using universal primers (Ba515Rmod1, Ar915aF and Reg1302R) for the three groups of microorganisms [32]. All primers contained the 454 Life

Science (Branford, CT, USA) adaptors A (5′-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3′) or B (5′-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3′) for Titanium sequencing, and a unique 12-base error-correcting barcode was attached to adaptor A for sample identification [33,34]. A PCR master mix of 76 µl was prepared for each DNA sample (per microbial group), as previously described [32]. An aliquot of 19 µl was transferred to serve as a no-template negative control. The remaining 57 µl of reaction mix were spiked with 10–40 ng of DNA contained in 3 µl of water, and then divided into three aliquots of 20 µl each. Amplification was performed in a Mastercycler proS (Eppendorf, Hamburg, Germany) using a previously described protocol [32]. Triplicate PCR products were pooled, and correct sizes of PCR products and signal absence from the negative controls were verified by agarose gel electrophoresis.

Following quantification of PCR products using the Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and a fluorometer (BioTek Instruments, Winooski, VT, USA), amplicons of the same target gene and region were pooled into three separate pools and loaded onto a 1%-agarose gel (wt:vol) prepared with 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8 with NaOH). Bands were visualised under blue light transillumination, excised, and DNA purified from the gel slices with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Gel-purified amplicon pools were quantified in triplicate with the Quant-iT dsDNA HS assay kit (Invitrogen). The three amplicon pools were normalized to contain 1×10^9 copies μl^{-1} and subsequently mixed at a ratio of 5:1:1 (Bacteria, Archaea, Protozoa) [32]. This final pool was sent to MWG Eurofins (Ebersberg, Germany) for Titanium pyrosequencing on a 454 Life Sciences Genome Sequencer FLX machine (454 Life Sciences, Branford, CT, USA).

Phylogenetic analysis of pyrosequencing reads

Samples were processed and analysed using the software QIIME (Quantitative Insights Into Microbial Ecology) v1.5 [35]. Sequence reads were assigned to corresponding samples by examining the 12-nucleotide error-correcting Golay barcodes using the split_libraries.py script in QIIME. The split_libraries.py script (with default settings) selected sequences that had a minimum average quality score of 25, a maximum of six ambiguous bases with no allowance for mismatches in primer sequence, a maximum homopolymer run length of six and a maximum sequence length of 1000 bp. All sequences that did not meet the quality-filtering criteria were excluded from downstream analysis. For bacteria and archaea, only sequences that were ≥ 400 bp in length (including primers, option -l), and in which both the forward and reverse primers were detected, were selected for further analyses. The primers were subsequently removed (option -z truncate_remove), and this option allowed only high-quality sequences to be retained, in which the reverse primer sequence was unambiguously detected. The remaining sequences that did not meet the quality criteria were removed from the bacterial and archaeal sequence libraries. Bacterial and archaeal 16S rRNA gene sequence data were denoised using Acacia [36] and chimera checked using the blast fragment method in QIIME [35,37] against the Greengenes database (gg_13_5/gg_13_5.fasta [38]). All sequences that did not meet the quality criteria, and those identified as pyrosequencing noise or potential chimeras, were removed from the bacterial and archaeal libraries used for further downstream analysis. Sequences stemming from ciliate 18S rRNA genes that were >200 bp in length were truncated to variable lengths so that the average quality score was >25 (all other parameters were set on the default option). The remaining sequences that did not meet the quality criteria were removed from the ciliate protozoal library.

Clustering of operational taxonomic units (OTUs) was performed using the uclust method [39] for bacteria and archaea at a 97% similarity threshold, or the prefix-suffix method passing the option "-p 1000" for protozoa (QIIME team, unpublished). Representative OTUs were assigned to taxonomic ranks as follows: bacterial 16S rRNA genes were BLAST-searched against the Greengenes database (gg_13_5/gg_13_5.fasta [38]); archaeal 16S rRNA genes and protozoal 18S rRNA genes were BLAST-searched against a rumen specific, in-house database [40] and the Silva eukaryotes database v.111 [41], respectively.

Of the samples collected in the study, 71/72 samples had at least 1000 bacterial sequences per sample. The remaining one sample had 268 sequences and was removed from the bacterial library used for further downstream data analysis. Within the archaeal library, 70/72 samples had at least 320 sequences per sample. The remaining two samples, which had 15 and 236 sequences, were removed from the archaeal library used for downstream data analysis. Since PCR amplicons for ciliate 18S rRNA genes were obtained from only 36/72 DNA samples, we used a minimum sequence read cut-off of 250 sequences per sample to report ciliate diversity in 26/36 samples. Subsequently, the OTU tables were rarefied at 1,000 (bacteria), 320 (archaea) and 250 (ciliate protozoa) sequences per sample and relative abundance tables were obtained at the phylum-, family- and genus-levels (bacteria), a mixed-taxon-level (archaea), or genus-level (ciliate protozoa).

To access the richness of microbial species captured within the samples, collector's curves for bacteria, archaea and ciliate protozoa communities were constructed from the OTU tables generated in QIIME, by using the alpha_diversity.py script and the observed species metric. The alpha-diversity rarefaction analysis was computed for 1000 sequences per sample for bacteria which included 71/72 samples, 320 sequences for archaea (including 70/72 samples) and 250 sequences for ciliate protozoa (including 26/36 samples). The collector's curves for the three microbial groups were visualised in SigmaPlot (2008 version 11, Systat Software, Inc., San Jose, CA, USA) by plotting the mean number + SD of OTUs observed against the number of sequences sampled.

When considering faecal samples from Groups A and B within each microbial group, bacterial phyla with relative abundances < 1% in all samples were grouped as "Other Phyla". Similarly, bacterial families or genera with relative abundances <1% in all samples were grouped as "Other Families" or "Other Genera", respectively. The archaeal community was categorised at a mixed-taxon level, and archaeal clades with relative abundances <1% in all samples were grouped as "Other Taxa". The ciliate protozoa community was categorised at genus level and all ciliate protozoa genera with relative abundances <1% in all samples were grouped as "Other Genera". Sequence data generated in this study were deposited in the NCBI SRA under study accession number SRP033608.

Statistical analyses

Alpha-diversity was evaluated at the OTU level using the QIIME pipeline [42]. The sampling completeness was evaluated by using the Good's coverage estimator, which calculates the probability that a randomly selected amplicon sequence from a sample has already been sequenced [43,44]. Good's coverage [43] was calculated in Excel (version 2010, Microsoft Corp., Redmond, WA, USA), and presented as mean percentage ± SD for each microbial group. Additional diversity indices (species richness,

species evenness, Shannon-Wiener's diversity index, Simpson's index of diversity) were calculated for the various levels (phylum, family and genus) for the bacterial community and a mixed-taxonomic level for the archaeal community, using the PAST software [45]. The species richness was evaluated by counting the number of taxa in the community and Pielou's species evenness was calculated to explain the biodiversity in each sample by quantifying the species equality based on the distribution of relative abundances of the species in the community (ranging from 0–1, where 1 was complete evenness with least variation in the community). The Shannon-Wiener diversity index [46] was computed to explain the entropy, taking into account the species richness and evenness of the community, which varied from 0 for communities with a single taxon, to high values of ~4.6 for highly diverse communities. Simpson's index of diversity (1-D) [47] was used to describe the diversity in a community, ranging from 0–1, with 1 indicating maximum diversity in a sample.

Beta-diversity was evaluated on a genus level for the bacterial community and a mixed-taxonomic level for the archaeal community, using the QIIME pipeline. Only microbial taxa that represented ≥1% of the total community, in at least one sample within each microbial group (bacteria and archaea), were included in the downstream analysis. Differences in bacterial and archaeal communities between samples were calculated using the Bray-Curtis (which takes into account the presence or absence of a species and the relative abundance) and Sørensen-Dice (which takes into account the presence or absence of a species) dissimilarity metrics. Principal coordinate analysis (PCoA) was performed in QIIME and the clustering of samples based on the first two principal coordinates was visualised in SigmaPlot. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was performed in QIIME, based on the Bray-Curtis dissimilarity matrix, to visualise the clustering of horses by diet on Days 0 and 4 of the study period, and the dendrograms were visualised in MEGA5 (version 5.2) [48].

The data generated in QIIME and PAST were imported into Excel, and re-formatted where necessary, before tests for statistical significance were conducted in STATA version 12.1 (Stata Corp, College Station, TX, USA). Non-normally distributed data are presented as median percentage and IQR throughout, and the non-parametric Kruskal-Wallis test was used to test for differences between the relative abundances of taxa identified in the faecal microbiomes of horses in Groups A and B. The level of significance used for differences between the bacterial and archaeal diversity indices on Days 0 and 4 was $P<0.017$ after Bonferroni adjustment for multiple comparisons. The relative abundances of bacterial communities between groups and within each group, on Days 0 and 4, were compared at multiple taxonomic levels (phylum, family and genus), with significance levels of $P<0.004$ (phylum level) and $P<0.001$ (family and genus levels) after Bonferroni adjustment for multiple comparisons. The relative abundances of the taxa in the archaeal communities between groups and within each group, on Days 0 and 4, were compared at a mixed-taxonomic level, with a significance level of $P<0.008$ after Bonferroni adjustment for multiple comparisons.

Inter-group (horses in Group A and B) and intra-group (horses in the same group) variation in bacterial and archaeal community structure on Day 0 were compared using the Bray-Curtis dissimilarity matrix to create a median value for each set of comparisons. The inter-horse (between horses in Group A and B on multiple sampling days) and intra-horse (horses compared with self on multiple sampling days) variation on Days 4–21 were also compared using Bray-Curtis dissimilarity to create a median value for each set of comparisons. The non-parametric Kruskal-Wallis test was used to test differences between the median values for each set of comparisons and the level of statistical significance was $P<0.05$.

Data for analysing the community structure of faecal microbiota (bacteria, archaea and ciliate protozoa) of pasture-fed horses are reported. The relative abundance data generated from QIIME were extracted, reformatted in excel to exclude the values for Day 0 in both Group A and B, for each microbial group, and the results presented as a median percentage and IQR. Diversity indices were calculated using the PAST software for the ciliate protozoa data for pasture-fed horses, after excluding the values for Day 0 in both Groups A and B, and presented as median and IQR.

Results

Amongst the prokaryote domains, PCR amplicons of bacteria and archaea were obtained from all 72 faecal samples. Despite repeated testing of variable dilutions of DNA template concentrations, PCR amplicons of ciliate protozoa were only obtained from half of the faecal samples (36/72), which was insufficient to adequately represent the two groups of horses on each sampling day. The results of the phylogenetic analysis for the ciliate protozoa are therefore described as part of the faecal microbiota of pasture-fed horses, but are excluded from downstream analysis involving diet-specific comparisons.

Metrics of pyrosequencing data for three microbial groups

The 72 faecal samples generated just under a million sequence reads (981,946) for the pooled microbial communities (bacteria, archaea and protozoa). Quality filtering and barcode mapping through the QIIME pipeline resulted in 553,715 sequences, the majority of which were, as expected from the pooling ratio, from the bacterial group (73%, 401,996), and the remaining sequences were archaea (71,403) and ciliate protozoa (80,316) (Table 1). Denoising of the bacterial and archaeal sequences resulted in 387,603 and 66,273 sequences respectively, and after removal of potential chimeras the number was further reduced to 387,083 and 65,639 sequences for bacteria and archaea respectively (Table 1). A total of 86,692 unique OTUs for bacteria and 63 unique OTUs for archaea were identified at 97% sequence similarity, from the total number of sequences obtained after chimera removal, and 36,896 unique OTUs were identified at 100% sequence similarity from all sequences for ciliate protozoa. The OTU tables for each microbial group were rarefied leaving a total number of 25,309 and 48 unique OTUs identified at 97% sequence similarity for bacteria and archaea, respectively, and 3,851 unique OTUs identified at 100% sequence similarity for the ciliate protozoa.

Within the domain Bacteria, 19 phyla were detected, which encompassed at least 93 different families and 158 different genera (Table S2A). In Groups A and B, just over half of the phyla (10/19) had relative abundances ≥1% in at least one sample and the remaining phyla (9/19) had relative abundances <1% in all samples (collectively referred to as "Other Phyla"). Two thirds of the families (65/93) had relative abundances <1% ("Other Families"), leaving a third of the families (28/93) with relative abundances ≥1% in at least one sample. The majority of genera (118/158) had relative abundances <1% ("Other Genera"), the remaining genera (40/158) had relative abundances ≥1% in at least on sample. Several organisms were detected, which are as yet "unclassified" in the Greengenes database. In the domain Archaea, two phyla were detected, encompassing at least five different families and 10 different clades (Table S2B). Ciliate

Table 1. Metrics of data generated by 454 GS FLX Titanium pyrosequencing of 16S and 18S rRNA gene amplicons from microbial groups present in 72 equine faecal samples.

Details	Microbial Group		
	Bacteria	Archaea	Ciliate protozoa
Sequences after quality-filtering			
Number of sequences	401,996	71,403	80,316
% of total sequences	72.6%	12.9%	14.5%
Mean number of sequences per sample	5,584	992	2171
(range)	(268–15,493)	(16–2,608)	(1–10,736)
Mean length of sequences	521.2	506.2	516.6
Mean length of sequences after removal of primers	470.8	452	483.6
Number of sequences after denoising	387,603	66,273	*
Number of sequences after chimera removal	387,083	65,639	*
Sequences after sub-sampling[&]			
Mean number of sequences per sample after sub-sampling	5,448	934	3,084
(range)	(1,333–15,006)	(322–2,438)	(252–10,736)
Number of sequences per sample (rarefied)	1000	320	250

*Not applicable (see Materials and Methods section).
[&]Samples with low number of sequences were excluded from the microbial libraries (see Materials and Methods section).
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protozoa were detected in 26 out of 72 samples and belonged to at least 15 different genera (Table S2C).

Rarefaction analysis and coverage of microbial diversity

Rarefaction analysis for the three microbial groups is presented in Figure 1. There was a plateau in the number of new OTUs detected when a minimum of approximately 320 sequences per sample and 250 sequences per sample were rarefied for the archaeal and ciliate protozoal groups, respectively. The collector's curve for the bacterial group had still not reached an asymptote when rarefied at a minimum of approximately 1,000 sequences per sample (Figure 1, S1A, B). However, Good's coverage estimates indicated that the sampling depth had adequately captured a large part of the species diversity in all three microbial groups, with the mean coverage being 99.60±0.17% for the bacterial community, 99.94±0.07% for the archaeal community, and 99.90±0.11% for the ciliate protozoal community.

Composition of the faecal bacterial community pre-dietary change

Diversity Indices. There was a difference in the median Simpson's indices of diversity (1-D) of bacterial genera in Group A (0.80 [IQR 0.79–0.82]) and Group B (0.85 [IQR 0.84–0.85]) ($P = 0.016$). There were no significant differences between Group A and B for the median Shannon-Wiener (2.22 [IQR 2.07–2.33] and 2.35 [IQR 2.28–2.38]; $P = 0.149$) and evenness (0.31 [IQR 0.28–0.31] and 0.38 [0.31–0.32]; $P = 0.423$) diversity indices.

Comparison of relative abundances of taxa in the bacterial community at multiple levels. Two phyla, namely the Firmicutes and Bacteroidetes dominated the bacterial community, with median relative abundances of 80% (IQR 67–84) and 18% (IQR 13–27) in Group A horses and 74% (IQR 70–74) and 24% (22–28) in Group B horses, respectively (Figure 2A). There were no significant differences detected among bacterial phyla between the two diet-groups (Table 2). Members of the family Ruminococcaceae had the highest median relative abundance in both Group A (47% [IQR 43–53]) and Group B (30% [IQR 25–33]), followed by members of the family Lachnospiraceae (12% [IQR 8–18] in Group A; 22% [IQR 18–26] in Group B) and members of as yet unclassified families within the orders Clostridiales (11% [IQR 10–13]; 16% [IQR 15–18]) and Bacteroidales (8% [5–10]; 9% [8–13]), each in Groups A and B, respectively (Figure 2B). For the as yet unclassified members within the order Clostridiales, there was a difference in the median relative abundances between Groups A and B ($P = 0.003$).

At genus level, the highest median relative abundances were of as yet unclassified members within the family Ruminococcaceae (40% [IQR 39–41; 28% [IQR 23–29]), unclassified members within the order Clostridiales (11% [IQR 10–13]; 16% [IQR 15–18]), unclassified members within the family Lachnospiraceae (10% [IQR 7–14]; 19% [IQR 16–24]), and unclassified members within the order Bacteroidales (8% [IQR 5–10]; 9% [IQR 8–13]), each in Groups A and B, respectively (Table 3; Figure 2C). There was a difference between Groups A and B in the relative abundances of as yet unclassified members within the order Clostridiales ($P = 0.003$) and within the family Lachnospiraceae ($P = 0.006$), and the less abundant genera CF231 ($P = 0.004$) and BF311 ($P = 0.003$) (Table 3, S3A).

Beta diversity. Principal coordinate analysis on genus level using Bray-Curtis dissimilarity (which takes into account presence and absence as well as relative abundance of a taxonomic group) revealed clustering of horses by dietary treatment group with more than half (53.3%) of the variation explained by PC1 and 17.3% of the variation explained by PC2 (Figure 3A). Similarly, UPGMA dendrograms showed distinct clusters of horses by diet (Figure 4). The median inter-group dissimilarity of faecal bacterial communities (0.298 [IQR 0.235–0.347]) was significantly higher ($P = 0.0004$) than the median intra-group dissimilarity (0.220 [IQR 0.180–0.265]) of horses on Day 0. Clustering by diet was not observed in UPGMA dendrograms using the Sørenson-Dice dissimilarity metric, which only takes into account presence and absence of a taxonomic group (data not shown).

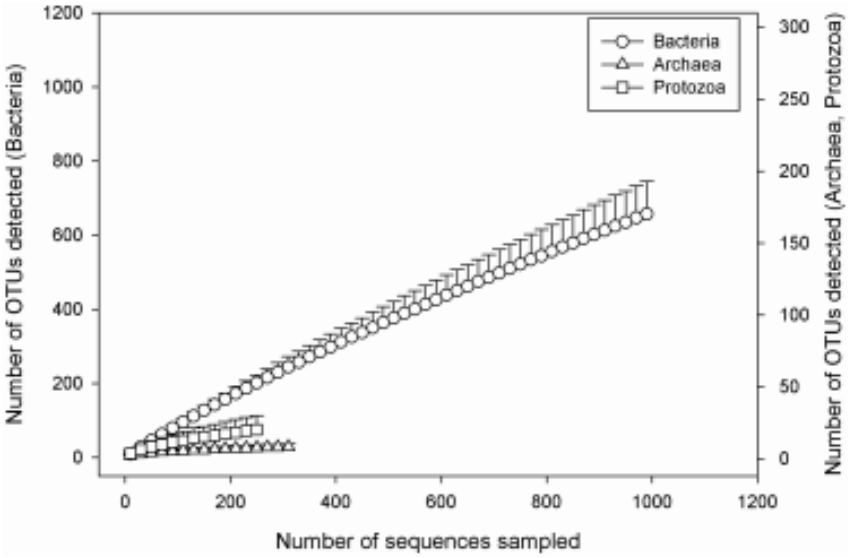

Figure 1. Rarefaction curves for microbial communities in faecal samples of forage-fed horses. The rarefaction curves show the mean number (with standard deviation) of observed species against the depth of sequencing of bacterial (-FONT NOT VALID-), archaeal (-Δ-) and ciliate protozoal (--) communities in the equine faeces sampled in the study (n = 72 faecal samples). Multiple rarefactions were calculated from the OTU tables obtained for each of the three microbial groups representing 71 out of 72 samples for bacteria (minimum of 1,000 sequence reads/sample), 70 out of 72 samples for archaea (minimum of 320 sequence reads/sample) and 26 out of 37 samples for ciliate protozoa (minimum of 250 sequence reads/sample).
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Dynamic adaptation of the faecal bacterial community post-dietary change

Diversity Indices. At genus level, the median Simpson's indices of diversity (1-D) were similar between bacterial communities in Groups A (0.87 [IQR 0.86–0.89]) and B (0.86 [IQR 0.85–0.87]) on Day 4 ($P = 0.425$). There were no significant differences in the median Shannon-Wiener (2.50 [IQR 2.43–2.56]; 2.43 [IQR 2.42–2.49]; $P = 0.335$) and the evenness (0.35 [IQR 0.32–0.38]; 0.33 [IQR 0.32–0.36]; $P = 0.262$) diversity indices between Groups A and B, respectively. There were significant differences in the median Simpson's indices of diversity of Group A horses between Day 0 (0.80 [IQR 0.79–0.82]) and Day 4 (0.87 [IQR 0.86–0.89]); ($P = 0.004$). The median Shannon-Wiener diversity indices of Group A horses on Day 0 (2.22 [IQR 2.07–2.33) and Day 4 (2.47 [IQR 2.43–2.56]) were significantly different ($P = 0.004$). There were no differences in the evenness diversity indices of Group A horses between Days 0 and 4. There were no significant differences in the diversity indices of Group B horses on Days 0 and 4 (data not shown).

Comparison of relative abundances of taxa in the bacterial community at multiple levels. The bacterial community was dominated by two phyla, Firmicutes and Bacteroidetes, with median relative abundances of 68% (IQR 63–75) and 28% (IQR 22–30) in Group A horses and 68% (IQR 62–73) and 27% (IQR 22–34) in Group B horses, respectively (Figure 2A). There were no significant differences between the relative abundances of bacterial phyla detected in the faeces of Group A and B horses on Day 4 (Table 2). Members of the family Ruminococcaceae had the highest relative abundance in both Group A (24% [IQR 20–27]) and Group B (26% [IQR 22–33]), followed by members of the family Lachnospiraceae (21% [IQR 17–25] in Group A; 20% [IQR 19–22] in Group B), members of as yet unclassified families within the orders Bacteroidales (16% [IQR 13–18]; 13% [IQR 12–18]) and Clostridiales (15% [IQR 13–17]; 15% [IQR 13–16]), each in Groups A and B, respectively (Figure 2B). There were no significant differences between the relative abundances of bacterial families detected in the faeces of Groups A and B horses on Day 4 (data not shown).

At genus level, the highest relative abundances in Groups A and B were of as yet unclassified members within the family Ruminococcaceae, unclassified members within the family Lachnospiraceae, and unclassified members within the orders Bacteroidales and Clostridiales (Figure 2C, Table S3A). Only one genus (RFN20, belonging to the phylum Firmicutes, family Erysipelotrichaceae), present at a low abundance (<2%), showed a difference in relative abundance between Groups A and B on Day 4 ($P = 0.004$; Table S3A). When comparing the faecal bacterial community of Group A horses, the relative abundances of three bacterial genera (*Pseudoramibacter Eubacterium* and as yet unclassified members within the family Ruminococcaceae and within the order RB046 of the phylum Armatimonadetes) differed between Day 0 and Day 4 (Table 4, S3B). There were no significant differences between the bacterial genera detected in the faeces of Group B horses that were grazing pasture on both Days 0 and 4 (Table S3C).

Beta diversity. On Day 4, bacterial communities did not cluster by diet-group (Figure 3B). Furthermore, there was no clustering by group from Day 4 through to Day 21 (Figure 5). Diet-specific clusters were observed between the horses fed Diet F (Group A horses on Day 0) and those fed pasture (Groups A horses on Days 4–21 and Group B horses on Days 0–21), (Figure S4). Inter- and intra-horse variation across all horses on pasture (Days 4–21 of both groups) had a median dissimilarity of 0.236 (IQR

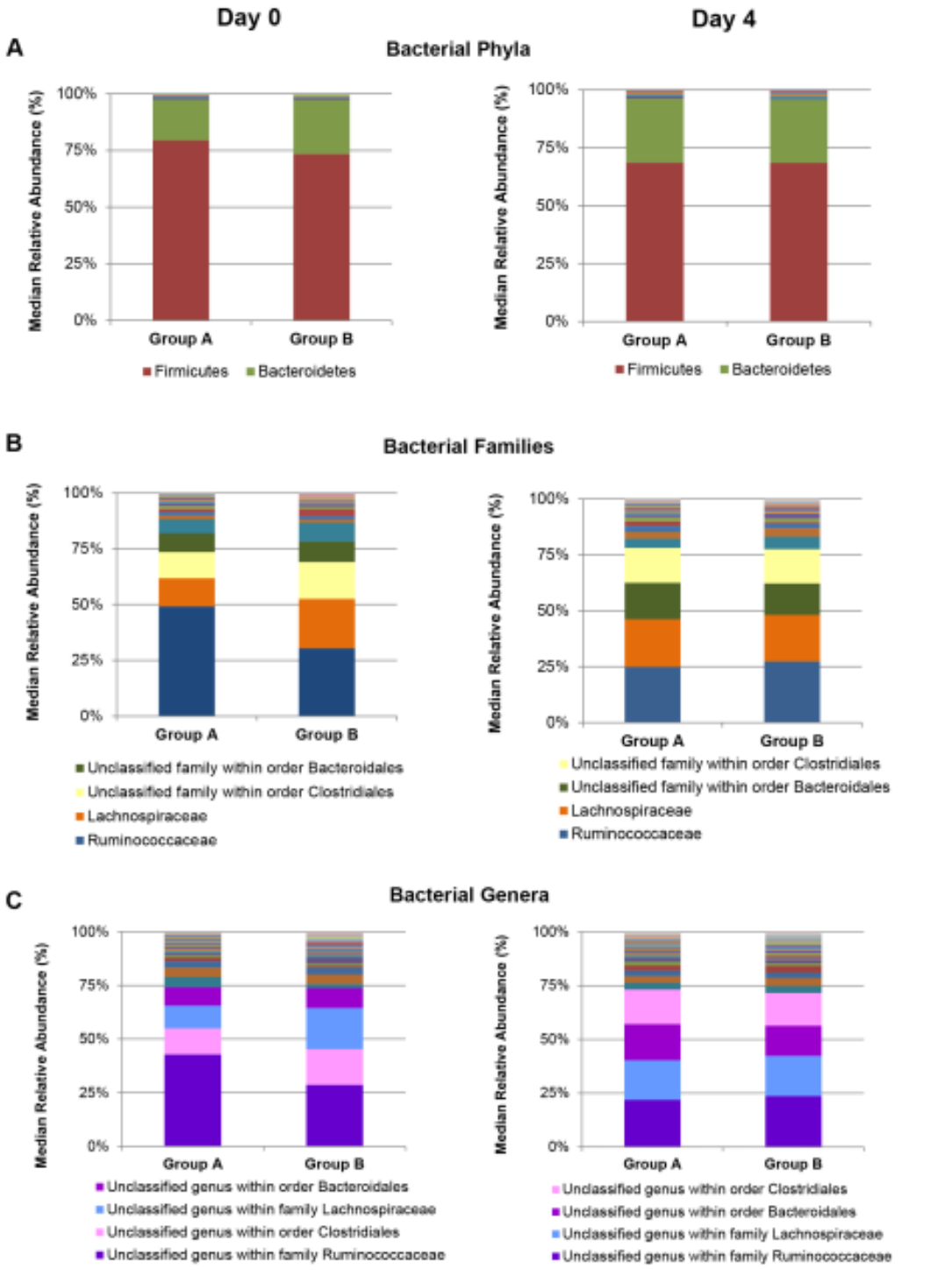

Figure 2. Comparison of the bacterial community structure at multiple levels. The median relative abundances of the bacterial phyla in the faeces of Group A and B horses, on Days 0 and 4, are illustrated in the figure, panel (A). Panel (B) shows the relative abundances of the bacterial families, and panel (C) shows the relative abundances of bacterial genera present in the faeces of Group A and B horses, on Days 0 and 4. The stacked

bar graph is presented in ascending order of the median relative abundances of bacterial taxa for Group A horses, and the legends show the most dominant taxa in each graph (>15% median relative abundance for the phylum level and >8% for the family and genus levels).
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0.189–0.291) and 0.222 (IQR 0.176–0.276), respectively ($P = 0.044$).

Composition of the faecal archaeal community pre- and post-dietary change

The median Simpson's indices of diversity were similar for both groups A (0.48 [IQR 0.19–0.55]; 0.42 [IQR 0.40–0.48]) and B (0.42 [IQR 0.36–0.46]; 0.45 [IQR 0.41–0.49]), on Days 0 and 4 respectively. The median Shannon-Wiener diversity indices for Group A (0.81 [IQR 0.44–0.95]; 0.70 [IQR 0.67–0.70]) and Group B (0.68 [IQR 0.60–0.72]; 0.70 [IQR 0.68–0.75]), and the evenness diversity indices for Group A (0.5 [IQR 0.31–0.61]; 0.59 [IQR 0.44–0.69]) and Group B (0.51 [IQR 0.45–0.68]; 0.54 [IQR 0.49–0.68]) on Days 0 and 4 respectively, were also similar. There were no significant differences in the diversity indices of the faecal archaeal community between Group A horses on Days 0 and 4, between Group B horses on Days 0 and 4, and between Group A and B horses on either Day 0 or Day 4 (data not shown).

Over half (6/10) of the archaeal clades in Groups A and B were present at a relative abundance of ≥1% in at least one sample, and the remaining clades were grouped as "Other Taxa". The archaeal community was dominated by two clades, *Methanocorpusculum* and relatives and *Methanobrevibacter ruminantium* and relatives in both groups, on both Day 0 and Day 4 (Figure S2, Table S4A, B). There were no significant differences in the median relative abundances of archaeal taxa between Group A horses on Days 0 and 4 (Table S4A), and Group B horses on Days 0 and 4 (Table S4B). There were no significant differences in the median relative abundances of archaeal taxa between Groups A and B on Day 0 or Day 4 (Table 5, S4C). Principal coordinate analysis did not show group-wise clustering of horses on Day 0 or on Days 4–21 (Figure S3). There was no significant difference in the inter-group or intra-group dissimilarity on Day 0 or on Days 4–21 ($P = 0.987$).

Composition of the faecal microbiota of horses maintained exclusively on pasture

The faecal microbiota of all horses (Groups A and B) grazing on pasture from Days 4–21 comprised of a diversity of bacteria, archaea and ciliate protozoa. The highest median relative abundances of bacterial taxa were of unclassified members within the family Ruminococcaceae (22% [IQR 17–27]), the order Bacteroidales (21% [IQR 17–25]), the order Clostridiales (12% [IQR 10–14]) and within the family Lachnospiraceae (11% [IQR 10–15]), which together contributed two-thirds (66%) of the bacterial community. The bacterial genera detected at >1% relative abundance were *Bacteroides*, *Blautia*, *BF311*, *CF231*, *Clostridium*, *Coprococcus*, *Fibrobacter*, *Mogibacterium*, *Oscillospira*, *Paludibacter*, *Parabacteroides*, *Prevotella*, *Pseudoramibacter eubacterium*, *p-75-a5*, *RFN20*, *Ruminococcus*, *Treponema and YRC22*. The microbiota of pasture-fed horses included 10 archaeal genera and their clades, six of which were present at ≥1% in at least one sample. The remaining four genera and clades (including *Methanobacterium*, *Sulfolobus* and relatives, *Methanobrevibacter arboriphilus* and relatives and *Methanosarcina* and relatives) were present at <1% relative abundance (collectively referred to as "Other Taxa"). The highest median relative abundances were of two clades, *Methanocorpusculum* and relatives (77% [IQR 58–90]) and *Methanobrevibacter ruminantium* and relatives (17% [IQR 8–39]), which together contributed 94% of the archaeal community in the faeces of pasture-fed horses. The remaining 6% was of the clade Rumen Cluster C and relatives (median relative abundance 3% [IQR 2–4]) and the genera *Methanosphera*, *Methanimicrococcus* and Other Taxa, each at < 1% median relative abundances.

Within the ciliate protozoa community (Table S2C), 15 genera under the class Litostomatea were identified as part of the faecal microbiota of pasture-fed horses. These included nine genera under the sub-class Trichostomatia present at ≥1% in at least one sample, and at least six other genera under the sub-classes Trichostomatia and Haptoria that were present at <1% relative abundance (collectively referred to as "Other Ciliate Genera"). The median Simpson's index of diversity (1-D) for the ciliate community of pasture-fed horses was 0.66 (IQR 0.48–0.73), and the diversity indices for Shannon-Wiener and evenness were 1.4 (IQR 0.9–1.5) and 0.49 (IQR 0.40–0.61), respectively. The highest median relative abundance was represented by the genus *Spirodinium* (24% [IQR 7–41]), followed by the genera *Triadinium* (9% [IQR 4–22]) and *Cochliatoxum* (6% [IQR 1–11]). The other genera identified were *Blepharocorys*, *Bundleia*, *Cyclopostbium* (each at ~4% median relative abundance) and *Isotricha*, *Polydiniella*, and the group of Other Ciliate Genera (including *Didinium*, *Epiplydium*, *Pelagodileptus*, *Diplodinium*, *Entodinium* and *Eremoplastron*) each at <1% median relative abundance.

Discussion

The present study investigated changes in the faecal microbiota via pyrosequencing of 16S rRNA gene amplicons derived from microbes present in the faeces of two groups of yearling horses. The results demonstrated dietary effects on the bacterial communities in the faeces, with changes occurring in their relative abundances within four days following dietary transition.

In the present study, group-wise clustering of bacterial community structures on Day 0 was observed only by using the Bray-Curtis dissimilarity metric (Figure 3A and 4) and not the Sørensen-Dice dissimilarity metric, indicating that the differences in community structure observed in the PCoA plots were due to differences in the relative abundances of the bacterial genera, and not the presence or absence of certain genera in the faecal samples from horses fed either of the two diets. Due to PCR bias [49], there are some limitations in the use of sequencing techniques to compare the true diversity of microbial communities, as there is a possibility that true abundances in an ecosystem may differ from those detected. However, based on current literature, it appears fair to compare relative abundances of microbial populations in samples that have been processed equally. Therefore, it is reasonable to conclude that the initial differences observed between the faecal microbiota of horses in the two groups of the present experiment were linked to nutritional/dietary factors, given the convergence of the microbial community structure once on similar pasture diets.

The median Simpson's index of diversity for the bacterial community in the faeces of horses fed the ensiled conserved forage-grain diet differed significantly from that of horses on pasture, and upon dietary transition, the diversity increased rapidly from 0.80 to 0.87 within four days of feeding on pasture. A high diversity in the microbiota may be a natural evolutionary

Table 2. Comparison of the relative abundances of bacterial phyla in the faeces of Group A (fed Diet F, n = 6) and Group B (fed Diet P, n = 6) horses on Day 0, and Day 4 (both groups fed Diet P).

	Relative abundance on Day 0					Relative abundance on Day 4				
	Group A		Group B			Group A		Group B		
Bacterial Phylum	Median	IQR[a]	Median	IQR[a]	*P*-value[b]	Median	IQR[a]	Median	IQR[a]	*P*-value[b]
Firmicutes	0.797	0.669–0.838	0.736	0.704–0.740	0.420	0.684	0.631–0.750	0.680	0.621–0.728	1.000
Bacteroidetes	0.181	0.131–0.273	0.240	0.216–0.278	0.260	0.276	0.222–0.298	0.272	0.223–0.335	0.870
Spirochaetes	0.006	0.004–0.007	0.003	0.002–0.004	0.140	0.004	0.002–0.005	0.008	0.004–0.010	0.060
Cyanobacteria	0.004	0.001–0.004	0.001	0.000–0.002	0.120	0.002	0.000–0.002	0.002	0.001–0.007	0.420
Fibrobacteres	0.003	0.002–0.005	0.005	0.003–0.009	0.200	0.008	0.004–0.010	0.010	0.008–0.017	0.200
Proteobacteria	0.003	0.002–0.004	0.003	0.001–0.004	0.800	0.003	0.002–0.005	0.002	0.001–0.003	0.170
Actinobacteria	0.002	0.001–0.004	0.009	0.007–0.021	0.010	0.008	0.006–0.009	0.012	0.010–0.012	0.020
Armatimonadetes	0.001	0.000–0.001	0.002	0.001–0.003	0.130	0.009	0.006–0.017	0.002	0.001–0.002	0.010
Planctomycetes	0.001	0.000–0.003	0.000	0.000–0.000	0.180	0.001	0.000–0.002	0.001	0.000–0.001	0.470
Synergistetes	0.000	0.000–0.002	0.000	0.000–0.001	0.700	0.001	0.000–0.001	0.000	0.000–0.001	0.630
Other Taxa	0.008	0.005–0.008	0.005	0.004–0.005	0.030	0.004	0.003–0.005	0.006	0.003–0.006	0.470

The bacterial phyla are listed in descending order of relative abundances for Group A horses on Day 0, and all phyla present at relative abundances of <1% in all samples are grouped as Other Taxa.

[a]IQR–Interquartile range.

[b]Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.004$.

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Table 3. Comparison of the relative abundances of bacterial genera in the faeces of Group A (fed Diet F, n = 6) and Group B (fed Diet P, n = 6) horses on Day 0.

	Relative abundance on Day 0				
Taxonomic rank within the domain Bacteria	**Group A**		**Group B**		
Phylum > Class > Order > Family > Genus	**Median**	**IQR[a]**	**Median**	**IQR[a]**	***P*-Value[b]**
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.395	0.387–0.413	0.281	0.227–0.293	0.016
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.112	0.103–0.131	0.163	0.149–0.181	0.003*
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.098	0.067–0.139	0.188	0.157–0.238	0.006*
Bacteroidetes > Bacteroidia > Bacteroidales > unclassified > unclassified	0.079	0.053–0.100	0.091	0.080–0.132	0.688
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.040	0.006–0.063	0.043	0.037–0.067	0.470
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.045	0.028–0.058	0.019	0.015–0.030	0.054
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > CF231	0.002	0.000–0.003	0.012	0.008–0.022	0.004*
Bacteroidetes > Bacteroidia > Bacteroidales > Bacteroidaceae > BF311	0.001	0.000–0.001	0.010	0.009–0.014	0.003*

The table lists bacterial genera that were present at relative abundances of ≥4% in both Groups A and B, and certain genera (present at <4% relative abundance) that were different between Groups A and B on Day 0. The taxonomic ranks are listed from Phylum to Genus in descending order of relative abundances for Group A.
[a]IQR–Interquartile range.
[b]Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$.
*Differences between Groups A and B.
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strategy for survival of a cursorial herbivorous browser, providing the opportunity to rapidly respond to varying quantities and types of forage available at different time-points. Annual and seasonal variation has been shown to occur in the growth and composition of the rye-clover mix pasture, and the water-soluble carbohydrate content in the grass may change diurnally [50]. The moderate fluctuation in the bacterial community structure observed in the horses maintained on pasture in the present study may be a result of changes in the composition of pasture and warrants further investigation (Figure 5).

The transition, from different bacterial community structures at the start of the experiment to similar community structures when both groups were maintained on pasture, occurred rapidly within a 4-day period. This was a shorter transition time than the six days post-dietary change in faecal samples previously reported [23], and may be due to the use of a more sensitive technique in the present study. The dietary challenge in the present trial was moderate and primarily forage-based. The rapid change observed in the structure of the faecal bacterial community when horses were transitioned from feeding on an ensiled conserved forage-grain diet to pasture, and the subtle changes in the bacterial community structure observed when on pasture, emphasizes the sensitivity of the microbiota to changes in dietary substrate. Given the results of the present study, it may be suggested that the basal

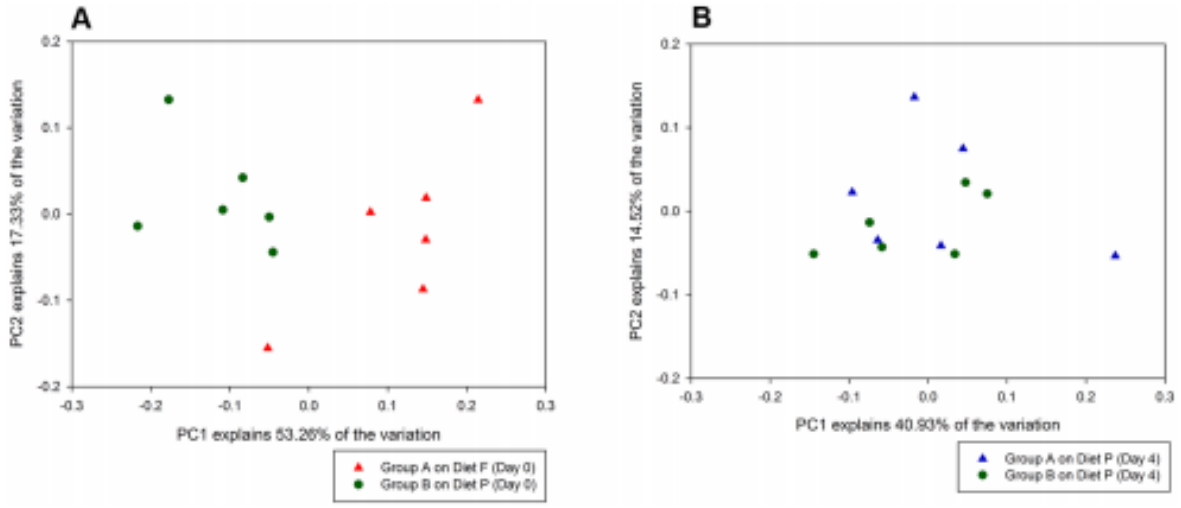

Figure 3. Principal coordinate analysis (PCoA) of bacterial communities detected in equine faeces pre- and post-dietary change based on the Bray-Curtis dissimilarity metric. (A) PCoA plot of faecal bacterial communities for Group A (Diet F) and Group B (Diet P) horses illustrates clustering of horses by dietary treatment group at Day 0. (B) PCoA plot of faecal bacterial communities for Group A (transition from diet F to diet P) and Group B (Diet P) horses illustrates no clustering by dietary treatment group at four days post-dietary change. Explanations for the symbols used are given in the legend.
doi:10.1371/journal.pone.0112846.g003

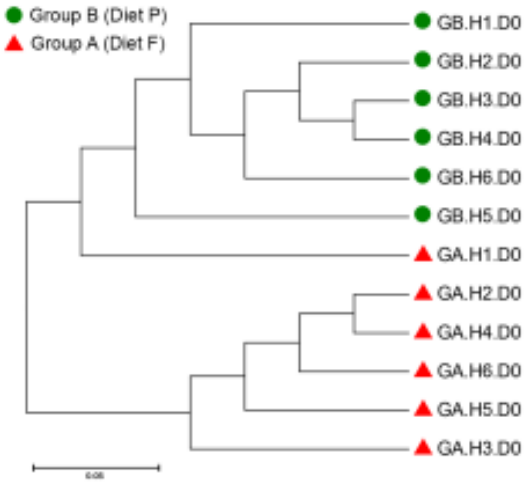

Figure 4. UPGMA dendrogram based on the Bray-Curtis dissimilarity matrix on Day 0. The Bray-Curtis dissimilarity metric takes into account the presence and absence of taxa and the relative abundances of the taxa, to generate a dissimilarity matrix for the faecal microbiota present in samples from horses on Day 0. The UPGMA dendrogram shows distinct clustering of horses (H; numbered from 1–6 per group) by group, indicating that the faecal bacterial community structure of horses on Day 0 differed between Group A (GA) and Group B (GB), which were fed Diet F and P respectively.
doi:10.1371/journal.pone.0112846.g004

microbiota that inhabits the horse's gastrointestinal tract is highly diverse and primed to respond to acute changes in diet. Hence, a shorter more intense sampling period around the time of dietary transition should be considered for future studies on the faecal microbiota of horses.

Bacteria are reported to form the largest proportion of the equine hindgut and faecal microbiomes [16] with the bacterial community dominated by the phyla Firmicutes and Bacteroidetes [13,15], which was also observed in the present study. At Day 0, there were no differences between Group A and Group B in the beta-diversity of the bacterial communities at the phylum level; however, there were differences at higher taxonomic resolution (at family and genus levels) (Figure 2). This finding of differences in the beta-diversity at family and genus levels is in contrast to the findings of other studies where a mixed population of horses were fed a variety of diets [13,14,22], and may be due to the higher resolution possible with the use of the next generation sequencing technique.

The number of unclassified bacterial genera (68) detected in the present study demonstrates the paucity of knowledge on the composition of the microbiota (Table S2A). Therefore, further cultivation and non-cultivation based studies in various populations of horses are required to evaluate the abundance and occurrence of the as yet unclassified organisms and to understand their functional role in hindgut microbial fermentation.

Lactic acid bacteria of the genera *Lactobacillus* and *Streptococcus* are potentially associated with grain diets and have been widely reported within the literature [8,51]. These genera have been implicated in the development of gastrointestinal disturbances and laminitis [13,19,20]. In the present study, the genus *Lactobacillus* was present at <1% relative abundance in the faecal microbiomes, whereas the genus *Streptococcus* was not detected in either group of horses, not even at relative abundances of <1%. Although a higher abundance of these genera may be detected in proximal regions of the hindgut than in the faeces, our findings are in contrast to many other reports in the literature (analysing caecal, colonic and faecal samples) when horses were fed grain: forage combinations or different types of forages [9,21,22,23]. None of the horses in the present study showed signs of colic, laminitis, or digestive disturbances throughout the study period, or the three weeks prior to Day 0. Given that grain diets are associated with shifts in microbial populations [52], the low abundance or non-detection of the lactic acid bacteria in the present study could be related to the dietary history of the yearling horses, wherein the management was primarily pasture-based and large quantities of grain had not been offered. There are indications that providing good quality pasture and increased activity may play a role in buffering the negative effects of grain

Table 4. Comparison of the relative abundances of bacterial genera in the faeces of Group A horses on Day 0 (fed Diet F, n = 6) and Day 4 (fed Diet P, n = 6).

Taxonomic rank within the domain Bacteria	Relative abundance of Group A horses				
Phylum > Class > Order > Family > Genus	Day 0		Day 4		
	Median	IQR[a]	Median	IQR[a]	*P*-Value[b]
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > unclassified	0.395	0.387–0.413	0.205	0.171–0.220	0.004*
Firmicutes > Clostridia > Clostridiales > unclassified > unclassified	0.112	0.103–0.131	0.151	0.130–0.167	0.078
Firmicutes > Clostridia > Clostridiales > Lachnospiraceae > unclassified	0.098	0.067–0.139	0.173	0.151–0.195	0.025
Bacteroidetes > Bacteroidia > Bacteroidales > unclassified > unclassified	0.079	0.053–0.100	0.158	0.132–0.177	0.109
Firmicutes > Clostridia > Clostridiales > Ruminococcaceae > *Ruminococcus*	0.045	0.028–0.058	0.025	0.018–0.032	0.146
Bacteroidetes > Bacteroidia > Bacteroidales > [Paraprevotellaceae] > YRC22	0.040	0.006–0.063	0.023	0.017–0.025	0.749
Firmicutes > Clostridia > Clostridiales > Eubacteriaceae > *Pseudoramibacter_Eubacterium*	0.001	0.000–0.002	0.006	0.004–0.007	0.004*
Armatimonadetes > SJA-176> RB046> unclassified > unclassified	0.001	0.000–0.001	0.009	0.006–0.017	0.005*

The table lists bacterial genera that were present at relative abundances of ≥4% in the faeces of Group A horses, and certain genera (present at <4% relative abundance) that were different between Days 0 and 4. The taxonomic ranks are listed from Phylum to Genus in descending order of relative abundances for Day 0.
[a]IQR–Interquartile range.
[b]Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.001$.
*Differences between Days 0 and 4.
doi:10.1371/journal.pone.0112846.t004

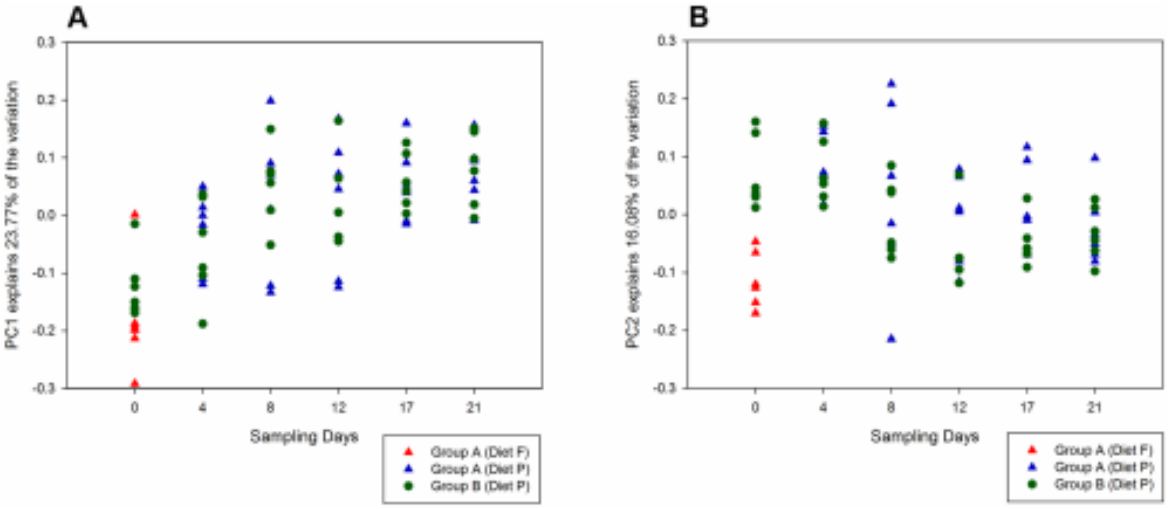

Figure 5. Principal coordinate analysis (PCoA) of bacterial communities detected in equine faeces over 3-weeks based on the Bray-Curtis dissimilarity metric. The plots illustrate the structure of the faecal bacterial communities in horses of Groups A and B on six sampling time-points over a period of three weeks. The plots show group-wise clustering of horses fed Diet F and Diet P on Day 0, and no clustering when horses were fed pasture (Diet P) from Days 4–21, with 24% variation explained on PC1 (A) and 16% variation explained on PC2 (B).
doi:10.1371/journal.pone.0112846.g005

supplementation on the hindgut microbiota [53]. It is not clear what quantity of grain or soluble carbohydrate content in pasture results in shifts in hindgut microbial populations, and this requires further investigation.

The present study targeted multiple domains of microorganisms and provided new information about the influence of diet on the structure of the Bacteria and other members of the eHGM, such as the archaea and ciliate protozoa. The diversity of the archaeal community was limited in the present study (Figure 1), and similar to previous studies [32,52], with sequence reads being assigned to only 10 different archaeal clades. The presence of the two archaeal clades (*Methanobrevibacter ruminantium* and relatives and *Methanocorpusculum* and relatives) in equine faeces has previously been reported [22,54], and these two clades dominated the archaeal microbiome of all horses in the present study (Figure S2). Analysis of the archaeal community of the horses did not show clustering by diet-group and none of the archaeal taxa detected were significantly more or less abundant in either of the treatment groups (Figure S3). These results indicate that the archaeal community structure was not influenced by the change in diet. This finding is aligned with the physiology of the archaeal organisms, which rely on the hydrogen produced by cellulolytic and other bacterial populations reported in the present study and only indirectly on the type of dietary substrate available to the host. However, this does not preclude that by using greater sample size or sequencing depth, impacts on the archaeal community may have been detected.

Table 5. Comparison of the relative abundances of archaeal taxa (clade-level) in the faeces of Group A (fed Diet F, n = 6) and Group B (fed Diet P, n = 6) horses on Day 0.

Taxonomic rank under the domain Archaea	Relative abundance on Day 0				
Phylum > Class > Order > Genus/Clade	Group A		Group B		
	Median	IQR[a]	Median	IQR[a]	*P*-Value[b]
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_ruminantium_and_relatives*	0.630	0.413–0.897	0.663	0.569–0.769	0.710
Euryarchaeota > Methanomicrobia > Methanomicrobiales > *Genera_incertae_sedis* > *Methanocorpusculum_and_relatives*	0.177	0.056–0.309	0.319	0.219–0.431	0.200
Euryarchaeota > Thermoplasmata > Thermoplasmatales > Rumen Cluster C_and_relatives	0.016	0.003–0.031	0.016	0.009–0.038	0.850
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanobrevibacter* > *Methanobrevibacter_gottschalkii_and_relatives*	0.014	0.000–0.028	0.000	0.000–0.003	0.120
Euryarchaeota > Methanobacteria > Methanobacteriales > Methanobacteriaceae > *Methanosphaera*	0.011	0.003–0.025	0.000	0.000–0.000	0.030
Other Taxa	0.000	0.000–0.000	0.000	0.000–0.000	0.360

[a]IQR–Interquartile range.
[b]Level of statistical significance after Bonferroni adjustment for multiple comparisons $P = 0.008$.
doi:10.1371/journal.pone.0112846.t005

In the present study, at least 15 different genera of ciliate protozoa were detected in the faeces of pasture-fed horses (Table S2C), of which the genera *Spirodinium* and *Triadinium* were the most abundant. In contrast, previous microscopy studies have reported that the highest percentage composition of ciliates identified in the faeces of horses, were of the genera *Bundleia*, *Blepharocorys*, and *Polymorphella* [55,56,57]. The faecal samples in these studies were collected from racehorses that were fed a mixed diet comprised of grain and forages, or from horses with an unknown dietary history; whereas the faecal samples in the present study were from horses fed exclusively on pasture. Faecal microscopic examination was not used in the present study and the ciliate protozoa were amplified from only 26/72 faecal samples. Hence, it was not possible to determine whether there were differences between the ciliate communities of the horses fed different forage-based diets on Day 0 of the study, which may require further investigation in a large number of samples.

The horses used in the present study were of similar age, sex, height, weight and body condition, and were maintained as cohorts in paddocks on the same farm. This is in contrast to most other studies that sampled heterogeneous populations of horses that differed in their feeding and management [13,15,22]. The study was not designed to separate the effects of housing from the dietary effects between Groups A and B prior to Day 0, and the change from a loose-box environment to pasture may have influenced the results. However, faecal microbiota are more likely to be influenced by acute changes in diet than more subtle changes in housing. Although the sample size for each group was small ($n = 6$), it was similar to sample sizes used in previous 454 pyrosequencing-based studies investigating the hindgut microbiome [13,22].

The faecal samples in the present study were collected carefully from the faecal mass and snap frozen within two minutes of defecation, thereby limiting the possibility of environmental contamination. A number of studies have demonstrated a strong inter-relationship between the microbial communities in the distal region of the hindgut (colon) and faeces [15,58,59]. This inter-relationship of microbial communities along the hindgut was reported to be greatest between samples from the right dorsal colon and the rectum/faeces [16]. Therefore, it is reasonable to suggest that the changes observed in the microbial populations of the faecal samples in the present study may be representative of changes occurring in the distal regions of the hindgut. The inter-variation between Groups A and B was higher than intra-variation, as demonstrated by the clustering of horses by treatment group on Day 0 (Figure 4), which allowed the identification of differences between the faecal microbiota of horses fed two different forage-based diets using a sample size of six horses per group.

Conclusions

The findings of the present study indicate that the faecal bacterial community of yearling horses is highly diverse and the relative abundances of individual taxa change rapidly in response to changes in diet. The faecal microbiota of horses on a conserved forage-grain diet were similar to that of horses fed pasture in terms of species richness and diversity, and the structure of the archaeal communities, but differed significantly in terms of the relative abundances of distinct bacterial families and genera. It is possible that daily changes in pasture composition affect the faecal microbiota of horses and this requires further investigation.

Supporting Information

Figure S1 Rarefaction curves for bacterial communities in the faeces of individual horses in Groups A and B at two time-points. The rarefaction curves show the number of observed species against the depth of sequencing of bacterial communities in the faecal samples from individual horses in Groups A and B on Day 0 (panel A) and Day 4 (panel B). The minimum depth of sequencing per sample for the bacterial group was 1000 sequence reads per sample.
(TIF)

Figure S2 Relative abundance of archaeal clades in the faecal microbial community of horses. The chart shows the median relative abundance of archaeal clades in the faeces of horses in Groups A and B, and indicates the dominance of two clades; *Methanocorpusculum* and relatives and *Methanobrevibacter ruminantium* and relatives. The colours in the figure legend show the archaeal clades with median relative abundances >15%.
(TIF)

Figure S3 Principal coordinate plot for data on the archaeal community structure in the faeces of horses across all sampling days based on Bray-Curtis dissimilarity. The plot illustrates the similarities in the faecal archaeal communities in the faeces of horses in Groups A and B on six sampling time-points over a period of three weeks. Clustering of horses by diet was not observed on Day 0, and no clustering was seen when horses were fed pasture (Diet P) from Days 4–21, with 84% of the variation explained by PC1.
(TIF)

Figure S4 3-dimensional plot of bacterial communities in all 72 samples. The graph contains the data of the bacterial communities in the faeces of horses in Groups A and B at six time-points over the 3-week period. The graph indicates that the horses fed pasture (Diet P, green), from both Groups A (Days 4–21) and B (Days 0–21), clustered separately from the horses fed ensiled conserved forage-grain (Diet F, red) on Day 0.
(TIF)

Table S1 Dry matter content and nutrient composition of the diets. A) Nutrient composition of the ensiled lucerne and cracked maize feed (Diet F) that was provided to the horses of Group A prior to Day 0. The nutrient composition is given on a Dry Matter basis, as provided by the manufacturers (Fiber Fresh Feeds Ltd., Reporoa, New Zealand). B) Nutrient composition (Dry Matter basis) of the standard New Zealand rye-clover mixed pasture (Diet P) that was available to Group B horses prior to Day 0 of the study, and Group A and B horses during the study (Days 0–21).
(PDF)

Table S2 The faecal microbiome of New Zealand Thoroughbred yearling horses fed Diet F (ensiled-forage-grain diet) and Diet P (rye-clover pasture) during the study. A) The faecal bacterial community, B) The faecal archaeal community, and C) The faecal ciliate protozoal community. The bacterial, archaeal, and ciliate protozoal taxa identified in the faeces of horses in the present study are listed in the table according to the taxonomic ranks assigned using the Greengenes database (version gg_13_5).
(PDF)

Table S3 Comparison of the relative abundances of bacterial taxa (genus-level) in the faeces of Group A and B horses on Days 0 and 4 of the study. A) Bacterial genera in the faeces of Group A (fed Diet F) and B horses (fed Diet P) on Day

0 and Day 4 (both groups fed Diet P). B) Bacterial genera in the faeces of Group A horses on Day 0 (fed Diet F) and Day 4 (fed Diet P). C) Bacterial genera in the faeces of Group B horses on Days 0 and 4 (fed Diet P).
(PDF)

Table S4 Comparison of the relative abundances of archaeal taxa (clade-level) in the faeces of Group A and B horses on Days 0 and 4 of the study. A) Group A horses fed two different diets in Day 0 (Diet F) and Day 4 (Diet P). B) Group B horses fed pasture (Diet P) on Days 0 and 4. C) Group A and B horses fed pasture (Diet P) on Day 4.
(PDF)

Acknowledgments

The authors thank Lana Bishop (Equine Science - Massey University) for conducting the preliminary study as part of her Masters project and for managing the horses prior to the study period. We are thankful to Michelle Kirk and Ivy Men (laboratory assistance), Henning Seedorf (assistance with denoising and chimera removal from sequencing data) and Gemma Henderson (provision of QIIME-compatible archaeal 16S rRNA gene database; all AgResearch) for their support. Equine research facilities were kindly provided by Lucy Waldron, Adrienne and John O'Brien (LWT Animal Nutrition Ltd. and NewMarket Lodge – Manawatu, New Zealand) and the horse feed for the preliminary study provided by Fiber Fresh Feeds Ltd. (Reporoa, New Zealand), for which we are grateful.

Author Contributions

Conceived and designed the experiments: KAF CWR EKG DGT. Performed the experiments: KAF DGT. Analyzed the data: KAF SK CFB. Contributed reagents/materials/analysis tools: KAF SK ENB. Wrote the paper: KAF SK CWR EKG CFB ENB DGT. Obtained funding: KAF ENB DGT. Designed and conducted the laboratory component of the study: KAF SK ENB.

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G-6. New Zealand Pony Clubs Association, I-Coach Newsletter (June 2015).

SPORT NEW ZEALAND

I - COACH

THE NEW ZEALAND PONY CLUB NZPCA

NZPCA Coaching Magazine **June 2015**

We recently had an email from a young lady, Karlette Fernandes who is studying at Massey University. She has been working on 'Equine Metabolic and Digestive Health' - The Equine Hindgut Microbiome. Now this all sounds very scientific, and it is, but Karlette studied ponies at NZPCA Pony Clubs around Massey in Palmerston North. She weighed them, measured them and assessed the body condition of Pony Club horses, mainly to identify the possibility of obesity and hoof conformation in relation to laminitis. The first study was presented and published at a scientific conference in June 2014, and the second study is due to be presented at a scientific conference at the end of June 2015.

G-7. List of research presentations.

1. Aspects of dietary management and the dynamics of faecal microbiota of horses and ponies (*Equus caballus*) in New Zealand. *Equine Research Group*. Massey University, Palmerston North, New Zealand (April 2013).
2. Horses ponies and the essential balance of tummy bugs.*Three Minute Thesis (3MT).* Massey University, Palmerston North, New Zealand (August 2013).
3. A cross-sectional survey of rider and horse demographics, and the feeding, health and management of Pony Club horses in New Zealand. *IVABS Colloquium.* Massey University, Palmerston North, New Zealand (June 2014).
4. A cross-sectional survey of rider and horse demographics, and the feeding, health and management of Pony Club horses in New Zealand. *NZSAP Annual Conference*, Napier, New Zealand (June 2014).
5. Diet and Microbiota. *Fiber Fresh Feeds Nutrition Workshop*. Massey University, Palmerston North, New Zealand (October 2014).
6. Body condition and morphometric measures of adiposity in a cohort of Pony Club horses and ponies in New Zealand. *NZSAP Annual Conference*, Dunedin, New Zealand (June 2015).
7. Seasonal variation in the faecal microbiota of mature adult horses maintained on pasture in New Zealand: Comparisons over a twelve-month period. *IVABS Colloquium.* Massey University, Palmerston North, New Zealand (July 2015).
8. Feeding behaviour and gastrointestinal transit times in Thoroughbred horses during dietary transition between four different forage-based diets. *IVABS Colloquium.* Massey University, Palmerston North, New Zealand (November 2015).

G-8. Statements of contribution to doctoral thesis containing publications.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: KARLETTE ANNE FERNANDES

Name/Title of Principal Supervisor: Associate Professor David G. Thomas

Name of Published Research Output and full reference:

Fernandes, K. A., Rogers, C. W., Gee, E. K., Bolwell, C. F. and Thomas, D. G. (2014). A cross-sectional survey of rider and horse demographics, and the feeding, health and management of Pony Club horses in New Zealand. Proceedings of the New Zealand Society of Animal Production. Napier, 74: 11-16.

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study and implemented the online survey, curated and analysed the data, drafted and revised the manuscript with input from supervisors and NZSAP reviewers. The candidate presented the research findings during the Young Scientist Award session at the New Zealand Society of Animal Production Conference in June 2014, and at the Massey University IVABS Colloquium in June 2014.

Karlette Anne Fernandes — Candidate's Signature — 25/08/2016 — Date

David Thomas — Digitally signed by David Thomas Date: 2016.08.24 08:57:24 +12'00' — Principal Supervisor's signature — 25/08/2016 — Date

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Name/Title of Principal Supervisor: Associate Professor David G. Thomas

Name of Published Research Output and full reference:

Fernandes, K. A., Rogers, C. W., Gee, E. K., Bolwell, C. F. and Thomas, D. G. (2015). Body condition and morphometric measures of adiposity in a cohort of Pony Club horses and ponies in New Zealand. Proceedings of the New Zealand Society of Animal Production. Dunedin, 75:195-199.

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- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed the study, organised the project and implemented the face-to-face survey, visited Pony Club events and collected the data, curated and analysed the data, drafted and revised the manuscript with input from supervisors and NZSAP reviewers. The candidate presented the research findings at the New Zealand Society of Animal Production Conference in June 2015.

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Name/Title of Principal Supervisor: Associate Professor David G. Thomas

Name of Published Research Output and full reference:

Fernandes, K. A., Kittelmann, S., Rogers, C. W., Gee, E. K., Bolwell, C. F., Bermingham, E. N. and Thomas, D. G. (2014). Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PLoS ONE, 9: e112846.

In which Chapter is the Published Work: Chapter 5

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- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed and implemented the study, collected pasture and faecal samples from the horses at the Stud farm, conducted laboratory analysis, analysed the data, drafted and revised the manuscript with input from supervisors and PLoS ONE reviewers. The candidate presented the research findings at the Equine Research Group meeting at Massey University in April 2013.

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Name of Candidate: KARLETTE ANNE FERNANDES

Name/Title of Principal Supervisor: Associate Professor David G. Thomas

Name of Published Research Output and full reference:

Fernandes, K. A., Gee, E. K., Rogers, C. W., Kittelmann, S., Biggs, P. J., Bermingham, E. N., Bolwell, C. F. and Thomas, D. G. (2016). Seasonal variation in the faecal microbiota of mature adult horses maintained on pasture in New Zealand. PLoS ONE, Submitted.

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed and implemented the study, collected the samples, conducted laboratory analysis, recorded, curated and analysed the data, drafted and revised the manuscript with input from supervisors, co-authors and PLoS ONE reviewers. The candidate presented the research findings at the Massey University IVABS Colloquium in July 2015.

Karlette Anne Fernandes — Digitally signed by Karlette Anne Fernandes

Candidate's Signature

25/08/2016

Date

David Thomas — Digitally signed by David Thomas Date: 2016.08.24 11:01:23 +12'00'

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Name of Candidate: KARLETTE ANNE FERNANDES

Name/Title of Principal Supervisor: Associate Professor David G. Thomas

Name of Published Research Output and full reference:

Fernandes, K. A., Rogers, C. W., Gee, E. K., Fitch, G., Bolwell, C. F., Kittelmann, S., Bermingham, E. N. and Thomas, D. G. (2016). Comparison of gastrointestinal transit times in stabled Thoroughbred horses during abrupt dietary transition between freshly cut pasture and three conserved forage-based diets. BMC Veterinary Research, Submitted.

In which Chapter is the Published Work: Chapter 7

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed and implemented the study, organised and managed the nutrition trial, collected the samples, conducted laboratory analysis, recorded, curated and analysed the data, drafted and revised the manuscript with input from supervisors and co-authors. The candidate presented the research findings at the Massey University IVABS Colloquium in November 2015.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: KARLETTE ANNE FERNANDES

Name/Title of Principal Supervisor: Associate Professor David G. Thomas

Name of Published Research Output and full reference:

Fernandes, K.A., Rogers, C.W., Gee, E.K., Kittelmann, S., Bolwell, C.F., Bermingham, E.N., Biggs, P.J. and Thomas, D.G. (2016). Resilience in the population dynamics of faecal microbiota in stabled Thoroughbred horses following abrupt dietary transition between freshly cut pasture and three forage-based diets. PLoS ONE, Submitted.

In which Chapter is the Published Work: Chapter 8

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed and implemented the study, organised and managed the nutrition trial, collected the samples, conducted laboratory analysis, recorded, curated and analysed the data, drafted and revised the manuscript with input from supervisors and co-authors.

Karlette Anne Fernandes — Candidate's Signature — 25/08/2016 — Date

David Thomas — Digitally signed by David Thomas Date: 2016.08.24 11:02:46 +1200 — Principal Supervisor's signature — 25/08/2016 — Date

GRS Version 3– 16 September 2011

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ASPECTS OF DIETARY MANAGEMENT AND DYNAMICS OF THE FAECAL MICROBIOTA OF HORSES AND PONIES (Equus caballus) IN NEW ZEALAND

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