Fibre fermentation in the ileum

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Voor Papa

Abstract

Recently, several studies have suggested that the microbes present in the ileum (i.e., the end of the small intestine) can ferment dietary fibre resulting in organic acid production and contribute to the overall gastrointestinal tract (GIT) fermentation. However, studying human ileal fermentation is challenging due to inaccessibility of the small intestine.

The aim was to validate a newly developed and optimised *in vivo*/*in vitro* ileal fermentation assay based on the growing pig as an animal model for human adults. After the assay was validated, this method was used to quantify ileal fermentation and compare this with large intestinal fermentation. In addition, the effect of diet on ileal fermentation and which factor was a greater contributor to *in vitro* ileal fermentation (inoculum or substrate) were studied.

Firstly, *in vitro* ileal organic matter (OM) fermentability was similar to *in vivo* fermentability in the conventional grown pig. Artificially rearing and inoculating young pigs with an infant faecal inoculum did not improve the model. Secondly, the ileal microbiota from pigs and human ileostomates was found to have similar *in vitro* OM fermentability and organic acid production for arabinogalactan, fructooligosaccharides and pectin, even though some differences were found in the ileal microbial community. Therefore, the *in vivo*/*in vitro* ileal fermentation assay using conventional pigs is a preferred and valid model for studying ileal fermentation in the adult human. It was found that ileal fermentation was quantitatively significant and similar in magnitude to hindgut fermentation when using this validated assay. However, the microbial community and organic acid production (mainly acetic acid) in the ileum differed. It was also found that partly replacing cellulose with more fermentable fibres in the diet affected the ileal microbial community and its fermentative capacity in growing pigs. Lastly, the substrate (i.e., different fibre sources) was found to have a greater effect on

ileal fermentation than the inoculum (i.e., different ileal microbiota obtained by feeding pigs different diets).

In conclusion, this work has demonstrated the quantitatively significant contribution of ileal fermentation to overall GIT fermentation, and that the *in vivo*/*in vitro* ileal fermentation assay using the growing pig is a valid assay for studying ileal fermentation in the adult human. Dietary intervention can be used to shape ileal microbiota and fermentation.

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List of abbreviations

AG	Arabinogalactan
AID	Apparent ileal digestibility
ANOVA	Analysis of variance
AR	Artificially reared
AR+	Artificially reared plus inoculated with infant faecal extract
BCFA	Branched-chain fatty acid
BW	Bodyweight
CEL	Cellulose
CFU	Colony-forming units
CoA	Coenzyme A
CP	Crude Protein
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
FFQ	Food frequency questionnaire
FOS	Fructooligosaccharides
GC	Gas Chromatography
GE	Gross energy
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GOS	Galactooligosaccharides
HCl	Hydrochloric acid
HEI	Healthy Eating Index
HFA	Human-flora-associated
HFD	Healthy Food Diversity
HITChip	Human Intestinal Tract Chip
IBD	Inflammatory bowel disease
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KF	Kiwifruit
OM	Organic matter
MDS	Mediterranean diet score
NA	Not applicable
ND	Not detected
NRC	National Research Council
NS	Not significant
NSP	Non-starch polysaccharides
PBS	Phosphate buffered saline

PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PND	Postnatal day
PSY	Psyllium
PYY	Peptide YY
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
RS	Resistant starch
SBS	Short bowel syndrome
SCFA	Short-chain fatty acid
SD	Standard deviation
SEM	Standard error of the mean
SFB	Segmented filamentous bacteria
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SIBO	Small intestinal bacterial overgrowth
SSB	Sugar-sweetened beverages
TIM	TNO intestinal Model
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UHT	Ultra-high temperature

Publications

Journal articles

- **Hoogeveen AME**, Moughan PJ, de Haas ES, Blatchford P, McNabb WC, Montoya CA. Ileal and hindgut fermentation in the growing pig fed a human-type diet. Brit J Nutr. 2020:1-27. (Chapter 5)
- **Hoogeveen AME,** Moughan PJ, Henare SJ, Schulze P, McNabb WC, Montoya CA. Type of dietary fibre is associated with changes in ileal and hindgut microbial communities in growing pigs and influences *in vitro* ileal and hindgut fermentation. J Nutr. 2021;151:2976-85. (Chapter 6)
- **Hoogeveen AME,** Moughan PJ, Hodgkinson SM, Stroebinger N, Yu W, Elizabeth A. Rettedal EA, McNabb WC, Montoya CA. *In vitro* ileal fermentation was affected more by the fibre source fermented than the ileal microbial community of growing pigs. (Submitted to Journal of Nutrition) (Chapter 7)

Conference presentations

- **Hoogeveen AME,** Moughan PJ, Cognée M, Henare SJ, Schulze P, and Montoya CA. Ileal fermentation of dietary fibres – using a combined *in vivo*/*in vitro* ileal fermentation assay. *Food Structures, Digestion and Health International Conference, October 2017, Sydney* (poster presentation)
- **Hoogeveen AME,** Moughan PJ, Cognée M, Henare SJ, Schulze P, and Montoya CA. Delivery of short-chain fatty acids to the ileum and hindgut through dietary fibre fermentation. *7th International conference Delivery of Functionality in Complex Food Systems, November 2017, Auckland* (poster presentation)

- **Hoogeveen AME,** Moughan PJ, Henare SJ, Cognée M, Schulze P, McNabb WC, Montoya CA. Fermentation of dietary fibre sources using combined *in vivo*/*in vitro* ileal and hindgut fermentation assays. 7th International Dietary Fibre Conference, June 2018, Rotterdam (poster presentation)
- **Hoogeveen AME,** Moughan PJ, Henare SJ, Cognée M, Schulze P, McNabb WC, Montoya CA. Ileal and hindgut fermentations of dietary fibre sources. *Riddet Institute Conference, July 2018, Wellington* (oral presentation)
- **Hoogeveen AME,** Moughan PJ, Henare SJ, Cognée M, Schulze P, McNabb WC, Montoya CA. The dietary fibre source influences ileal and hindgut fermentation – a study using combined *in vivo*/*in vitro* methodology. *Focus on Fibre and Food Monitoring Symposium, February 2019, Dunedin* (poster and oral presentation)
- **Hoogeveen AME,** Moughan PJ, McNabb WC, Montoya CA. Dietary interventions influence the ability of ileal microbiota to ferment dietary fibres – using a combined ileal *in vivo* and *in vitro* methodology. *6th International Conference on Food Digestion, April 2019, Granada* (poster presentation)
- **Hoogeveen AME,** Moughan PJ, McNabb WC, Hodgkinson SM, Rettedal EA, and Montoya CA. Fermentative capacity of the ileal microbiota can be modified through dietary interventions in a pig model. *Food Structures, Digestion and Health International Conference, October 2019,* Rotorua (oral presentation)
- **Hoogeveen AME,** Moughan PJ, de Haas E, Blatchford P, McNabb WC, and Montoya CA. The difference in ileal and caecal microbial composition did not influence organic matter fermentation but altered short-chain fatty acid production in the growing pig fed a human-type diet. *NZMS Annual Conference, November 2019* (poster presentation)

- **Hoogeveen AME,** Moughan PJ, McNabb WC, Hodgkinson SM, Rettedal EA, and Montoya CA. Ileal microbiota and their fermentative capacity can be modified through dietary intervention in a pig model. *NZMS Annual Conference, November 2019* (oral presentation)
- **Hoogeveen AME,** Moughan PJ, McNabb WC, and Montoya CA. The dietary protein source affects ileal microbiota and their fermentative capacity in a pig model of an adult human. *Riddet Institute Conference, April 2021, Wellington* (poster presentation)

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Chapter 1: General introduction

This chapter provides an overview of the background, research questions, and thesis approach.

1.1. Research background

Microbial fermentation in the gastrointestinal tract (GIT) is an important process in humans. During fermentation, undigested dietary material (e.g., fibres) and endogenous material (e.g., mucins) are degraded by the microbiota resident in the GIT lumen. As a result of fermentation, the microbiota produces microbial metabolites, such as short-chain fatty acids (SCFAs) and gasses (e.g., CO_2, CH_4, and H_2) [1]. These microbial metabolites can benefit the host, both locally in the GIT and systemically. For example, SCFAs can be used as an energy source for epithelial cells [2], and after absorption, SCFAs are involved in glucose homeostasis [3]. Due to its important metabolic function for the host, the GIT microbiota is sometimes referred to as the “forgotten organ” [4]. However, the current research into GIT microbiota and their fermentation focuses predominantly on the large intestine due to the large number of microbes found there [5]. In addition, it is believed that there is little fermentation in the upper GIT (i.e., stomach and small intestine) [6, 7].

However, a growing number of scientific publications suggest that there is a considerable number of microbes in the human upper GIT and these microbes can ferment dietary fibre and produce SCFAs [8-12]. Within the upper GIT, a greater and more diverse microbial population is found at the end of the small intestine (i.e., the ileum) [8]. In addition, the digesta have a longer transit time through the ileum than other sections of the small intestine. Therefore, it is suggested that the fermentation in the upper GIT occurs mainly in the ileum.

It is challenging to study human ileal fermentation, due to inaccessibility of the ileum. Therefore, a combined *in vivo*/*in vitro* methodology has been developed and optimised to study ileal fermentation [13]. In this methodology, the growing pig is used as an animal model for the human adult because of the high anatomical similarity of the upper GIT between pig and human [14]. However, this assay has not been adequately validated. Validation of this methodology would include comparing predicted (i.e., *in vitro*) and determined (i.e., *in vivo*) ileal OM fermentability in the pig and then comparing the microbiota and its fermentative capacity in ileal digesta from pigs and humans.

1.2. Research aims and questions

Based on the knowledge gaps identified in the literature, this PhD research programme aimed to investigate the fermentation of dietary fibre in the ileum by (i) validating the combined *in vivo*/*in vitro* ileal fermentation methodology, (ii) comparing ileal fermentation with hindgut fermentation, and (iii) investigating the effect of diet on ileal fermentation.

To support the research aims, the following research questions were formulated and studied:

- **RQ1:** Does inoculating pigs with an infant faecal extract and raising them in a hygienic non-farm environment provided with human foods improve the combined *in vivo*/*in vitro* ileal fermentation methodology?
- **RQ2:** Is the combined *in vivo*/*in vitro* methodology valid for determining ileal fermentation in pigs?
- **RQ3:** Is the combined *in vitro* methodology valid for determining the ileal fermentation of foods in human adults?

- **RQ4:** What is the difference between ileal and hindgut fermentation based on OM fermentability and organic acid production?
- **RQ5:** How do dietary interventions affect ileal fermentation, and how do these effects compare with hindgut fermentation?
- **RQ6:** How does the diet influence the ileal microbiota and its fermentative capacity?
- **RQ7:** How is *in vitro* ileal fermentation affected by the different inocula and substrates?

1.3. Research approach

The research followed a structured plan to answer the above research questions. In the first part of the overall study, experiments were conducted to test the validity of a recently developed combined *in vivo*/*in vitro* ileal fermentation assay. The second part of the research used this methodology to test the significance of ileal fermentation compared to hindgut fermentation and how diet affects ileal fermentation. The research is reported in five experimental chapters (Figure 1.1).

In **Chapter 2**, a comprehensive literature review covers aspects of the research topic. The objectives of this review were to understand: (i) the current understanding of GIT fermentation; (ii) the evidence and potential of small intestinal fermentation; (iii) the various methodologies to research GIT fermentation, their challenges, and suitability to study small intestinal fermentation. It was hypothesised that the microbiota in the human ileum makes a significant contribution to overall GIT fermentation, and like the hindgut fermentation, the diet influences ileal fermentation.

It was considered important to properly validate the combined *in vivo*/*in vitro* ileal fermentation methodology for the pig by comparing the *in vitro* ileal OM fermentability with *in vivo* disappearance of OM in the pig itself (**Chapter 3**). In addition, Chapter 3 aimed to

test whether rearing pigs artificially (i.e., in a more hygienic, non-farm environment and receiving a human-type diet) and inoculating piglets with an infant faecal extract in the first days of their life would improve the ileal fermentation assay. The work reported in **Chapter 4** aimed to validate the model against the adult human by comparing the *in vitro* ileal fermentation of different fibre substrates using ileal microbiota obtained from pigs with human ileostomates.

After the combined *in vivo*/*in vitro* ileal fermentation methodology was validated, it was applied to provide insight into the significance of ileal fermentation compared to hindgut fermentation in terms of organic matter (OM) fermentability and organic acid production using the combined *in vivo*/*in vitro* methodology (**Chapter 5**). However, in this chapter, the pigs received only one human-type diet. Therefore, in the study reported in **Chapter 6**, the pigs received three different semi-synthetic diets to assess the effect of diet on the ileal and hindgut fermentation in terms of OM fermentability and organic acid production. For the work discussed in **Chapter 7**, ileal digesta from pigs receiving a larger number of different diets were collected to test how the ileal microbiota fermented various dietary fibre substrates. This allowed the study of the effect of both inocula and substrate in *in vitro* ileal fermentation. All research findings are brought together and discussed in **Chapter 8**, and recommendations for future work are given.

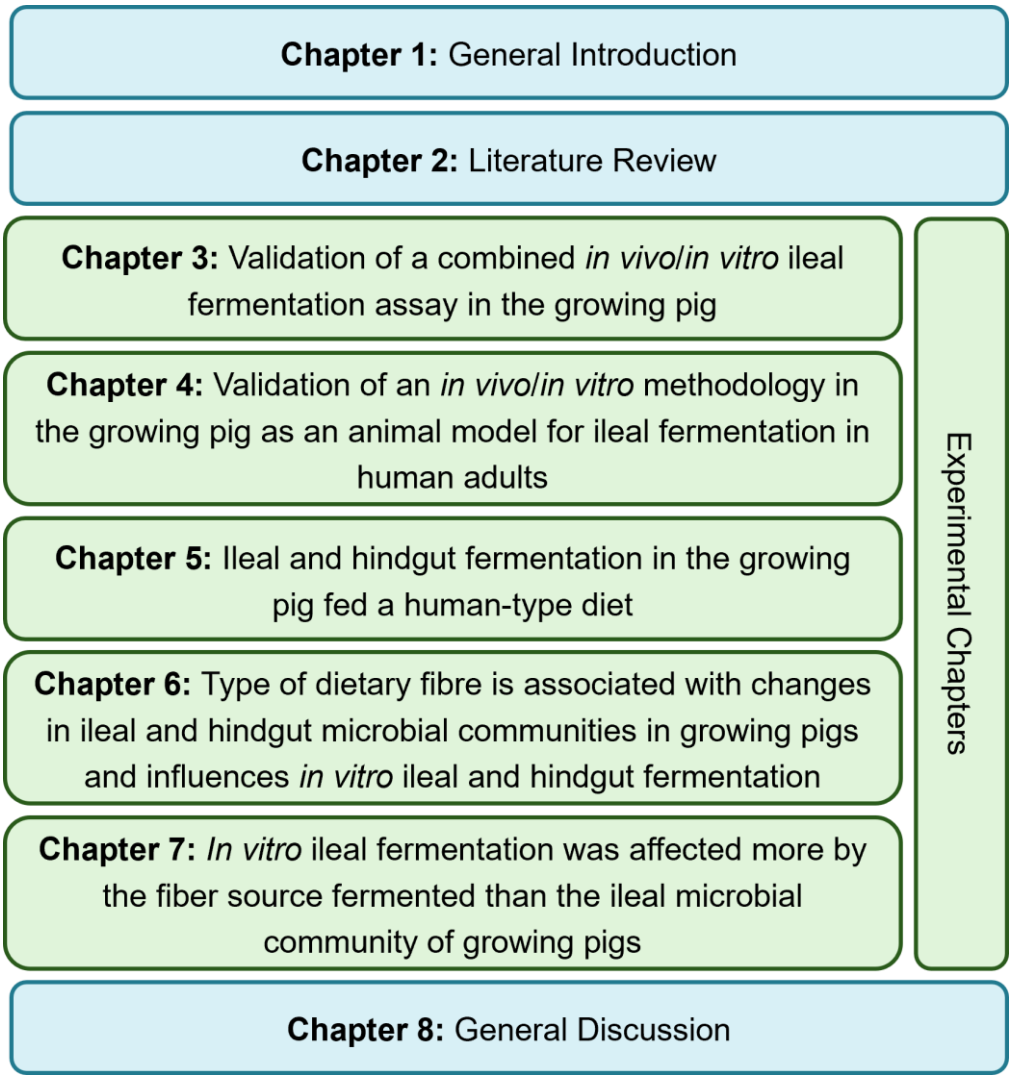

Figure 1.1: Thesis Layout.

Chapter 2: Literature review

2.1. Search strategy

For this literature review, relevant articles were searched from May 2017 to June 2022 via Scopus and Google Scholar. A search strategy was developed to get a better understanding of the current understanding of contribution of small intestinal microbiota to GIT fermentation (**Table 2.1**). Additional literature was identified by searching the bibliography in key articles and targeted searches for specific topics. Only literature published in English was included. The literature was filtered to include human and porcine studies (both *in vivo* and *in vitro*) and, to a lesser extent, murine or rat studies or studies.

Table 2.1: Keywords used to search relevant literature regarding microbial fermentation in the small intestine

Concept	Key words
Small intestine	Ileal, ileum, duodenum, jejunum, small intestine, upper gastrointestinal tract, upper gut, foregut
Fermentation	Ferment*, hydrolys*, breakdown, fibroly*
Fibre	Fibre, fibre, polysaccharide, prebiotic*, carbohydrate*
Microbiota	Microbio*, microbe*. bacteria

2.2. Gastrointestinal tract and digestion

The primary purpose of the human gastrointestinal tract (GIT) is to digest the consumed food and absorb the nutrients released during digestion. The GIT is divided into the mouth, stomach, and small and large intestines. Every section of the GIT is optimised to contribute to this primary function of the GIT. The different GIT sections are well studied, providing a good understanding of digestion in the human GIT (Figure 2.1).

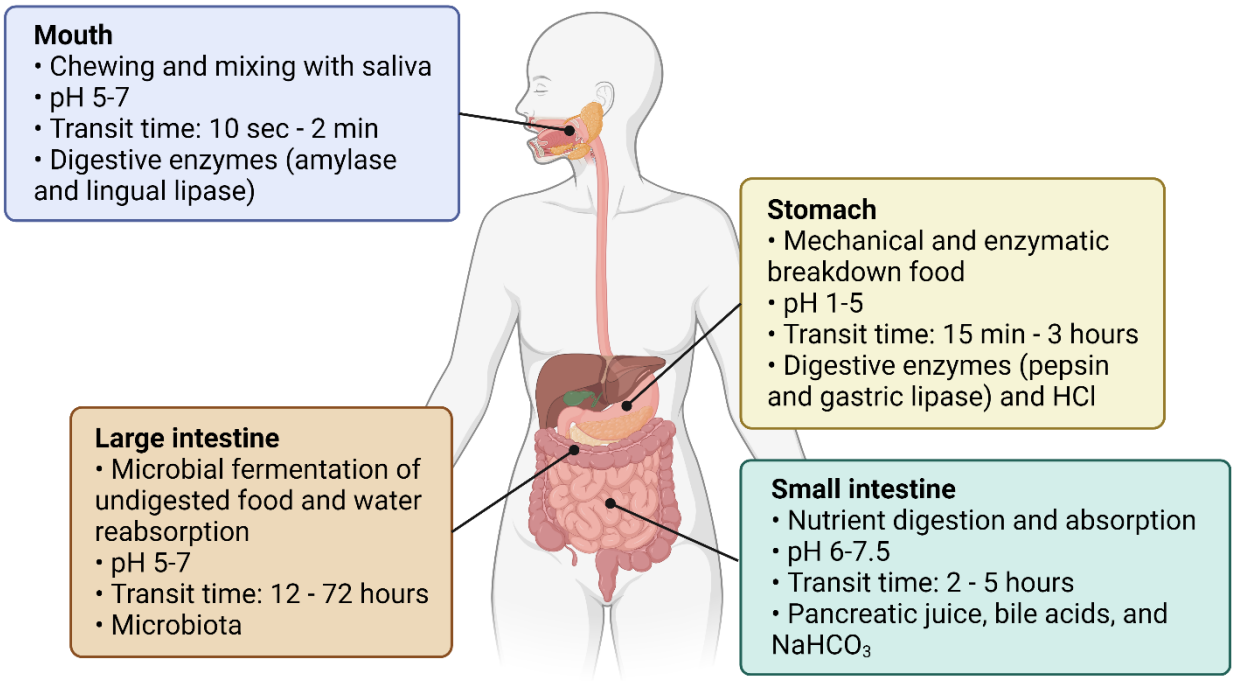

Figure 2.1: A schematic diagram of the different regions of the human gastrointestinal tract (GIT) and their main properties contributing to GIT digestion. Created with BioRender.com

The mouth reduces the particle size of the foods consumed by chewing and adds saliva containing digestive enzymes (i.e., α-amylase and lipase) to initiate food digestion. After swallowing, the bolus enters the stomach, adding more digestive enzymes (i.e., pepsin and lipase) and hydrochloric acid (HCl). HCl lowers the pH, which denatures proteins, increasing their digestion and inhibiting the growth of potential microbes ingested alongside the food. The small intestine is the main site of food digestion and nutrient absorption. Pancreatic juice, sodium bicarbonate, and bile acids are released into the small intestine. The pancreatic juice contains digestive enzymes, including lipase, proteases, and amylases. Sodium bicarbonate neutralizes the pH, and the bile acids assist in emulsifying lipids and forming micelles to promote fat absorption [15].

Some food remains undigested in the stomach and small intestine, mostly dietary fibre. The undigested food passes through to the large intestine, harbouring many microorganisms [5, 16]. These microbes can metabolise (i.e., ferment) the undigested dietary and non-dietary materials (e.g., mucin and shedded epithelial cells) to obtain energy, nitrogen, and sulphur for their growth and maintenance [1]. Some end-products of fermentation (e.g., butyrate) can be beneficial for humans (for example, as an energy source for epithelial cells) [17]. The passage of digesta through the large intestine is relatively slow compared to the upper GIT [18], which allows the bacteria enough time to metabolize most of the available nutrients.

Early research examining the GIT microbiota reported that the upper GIT (i.e. the stomach and small intestine) was virtually sterile [19]. In contrast, large numbers of bacteria were found within the large intestine and faeces [5, 16]. Therefore, a current belief commonly held is that microbial GIT fermentation predominantly occurs in the large intestine, with little to no fermentation in the upper GIT [6, 7]. However, since the development of molecular-based techniques to identify microbiota, many scientific publications have found considerable numbers of microbes in the human small intestine [12, 20, 21]. These microbes can ferment dietary fibre and produce metabolites [12]. The small intestinal microbiota may have a function in the digestion and fermentation of dietary and non-dietary materials before they reach the large intestine and could contribute to human health. However, the inaccessibility of the human small intestine makes researching the small intestinal microbiota and its fermentation challenging.

This review discusses (i) the current understanding of GIT microbiota and its fermentation, (ii) the evidence for small intestinal fermentation and its potential function, and (iii) the suitability of current *in vivo* and *in vitro* models used to study overall GIT fermentation to study small intestinal fermentation.

2.3. Microbial fermentation in the gastrointestinal tract

Microbial fermentation in the GIT is an anaerobic process whereby the GIT microbiota metabolizes dietary and non-dietary material. The microbiota secretes extracellular enzymes that can hydrolyse specific molecular bonds. These enzymes degrade carbohydrates and proteins into monomers (e.g., hexoses, pentoses, and amino acids), which can be taken in and further metabolised by the microbiota. The end products of fermentation include organic acids, such as short-chain fatty acids (SCFAs), and gases (e.g., methane, hydrogen, and carbon dioxide) [22]. Several health effects of GIT microbiota and fermentation are assigned to these end products. Most fermentation in the human GIT occurs in the ascending colon due to the high substrate concentration. It is reflected by the high SCFA concentrations [23, 24]. Due to fermentation, humans can obtain energy from undigestible dietary material in the form of SCFAs. Dietary fibres provide around 5% of the total daily energy requirements for humans [25]. The following sections describe (i) the available substrate of GIT fermentation, (ii) the biogeography of the human GIT microbiota, (iii) the fermentation products, and (iv) the effect of diet on GIT microbiota and its fermentation.

2.3.1. The available substrate for gastrointestinal tract fermentation

2.3.1.1. Dietary fibre

Dietary fibre is an important substrate available to the GIT microbiota. The Codex Alimentarius has defined dietary fibre as carbohydrates that are not digested by the host's enzymes [26]. Dietary fibres are a collection of polysaccharides with a degree of polymerization (DP) of more than ten monomers. These monomers can be pentoses (such

as arabinose and xylose) and hexoses (such as glucose and fructose) which are linked in a linear or branched fashion (Table 2.2). The only exception is lignin, which is an aromatic polymer. Some oligosaccharides (DP 3 to 10), such as fructooligosaccharides (FOS) and galactooligosaccharides, are also resistant to digestive enzymes and, therefore, classified as dietary fibres. Dietary fibres are known to have multiple beneficial effects on GIT physiology. For example, dietary fibres add bulk to the digesta, affecting transit time and digestion [27, 28].

Dietary fibres can be classified based on origin, chemical structure (e.g., side chain compositions and degree of esterification or acetylation), or physicochemical properties (e.g., solubility, viscosity, and overall GIT fermentability) (Table 2.2). The latter two characteristics affect the GIT microbiota and its fermentation. For example, pectin with a low degree of esterification is more quickly fermented than pectin with a higher degree of esterification [29]. The GIT microbiota can ferment soluble fibres more efficiently as soluble fibres disperse quickly in the aqueous GIT environment, making them more accessible to bacterial metabolism. An exception is psyllium, a soluble fibre that is not rapidly fermented [30]. Insoluble dietary fibres are more slowly fermented [31, 32].

Table 2.2: Common dietary fibres found in foods and their physiochemical properties. Adapted from Williams *et al.*, 2019 [33] and Gill *et al.*, 2021 [34][1]

Dietary fibre	Food source	Main unit	Branch units	Physicochemical properties		
				Solubility	Viscosity	Overall GIT fermentability
Cellulose	All plant cell walls	β-(1,4)-glucose	-	Insoluble	Non-viscous	Low
Lignin	All plant cell walls	Polyphenols	Polyphenols	Insoluble	Non-viscous	Low
β glucans	Oat, barley, fungi	β- (1,3) glucose	-	Low to medium	Medium	Medium to high
Hemicellulose						
Arabinoxylan	Cereal, psyllium[2]	Xylose	Arabinose	Low to medium	Medium	High[2]
Galactomannans	Guar gum, locust bean gum	Mannose	Galactose	Medium to high	Medium to high	High
Pectin	Fruit, vegetables, legumes	Galacturonic acid with methoxy groups	Arabinose Galactose	High	Medium to high	high
FOS/inulin	Cereals, fruit, vegetables	D-fructose with terminal glucose unit.	-	Medium to high	Medium to high	High
GOS	Milk and pulses	Galactose, with terminal glucose unit.	-	High	low	High
Resistant starch						
RS-1 (physically inaccessible	Whole grains, legumes, raw fruit vegetables	Amylose	Amylose	Insoluble	Non-viscous	High

RS-2 (starch confirmation)	Cereals, raw legumes, raw fruit, vegetables	Amylose	Amylose	Low	Non-viscous	High
RS-3 (retrograded)	Cooking and cooling of any starch source	Amylose	Amylose	Low	Non-viscous to low	High
RS-4 (chemically modified)	Synthesised	Amylose	Amylose	Low to high	Low to medium	High
RS-5 (starch-lipid complex)	Synthesised	Amylose	Amylose	Low	Low	Low

[1] FOS, fructooligosaccharides; GIT, gastrointestinal tract; GOS, galactooligosaccharides; RS, resistant starch.
[2] Due to their structural feature, psyllium sources of arabinoxylans are considered of only low fermentability.

2.3.1.2. Gastrointestinal tract endogenous material

Endogenous material (such as mucin, the host's digestive enzymes, and sloughed epithelial cells) can also provide a substrate for GIT fermentation. Mucins are glycoproteins produced by epithelial cells and function as a protective barrier. They contain high amounts of proline, threonine, and serine. They have carbohydrate side chains consisting of N-acetyl glucosamine, N-acetyl galactosamine, fucose, and galactose with a sialic acid or sulphate group at the end. Some GIT bacteria, such as *Akkermansia muciniphila,* are specialised in degrading these host mucins [35]. Due to the thicker mucosal layer, these specialised mucin-degraders are more abundant in the large intestine than in other GIT locations [36]. Other bacteria, such as *Bacteroides thetaiotaomicron*, can switch their metabolism to proteins when carbohydrates are deficient [37].

2.3.1.3. Microbial cross-feeding

Microbial cross-feeding is a phenomenon in the GIT whereby the intermediate or end-products of one bacterium become the substrate for another. The primary degraders within the human GIT microbiota are mainly Bacteroidetes which can metabolize various polysaccharides. For example, *Ruminococcus bromii* acts as a primary degrader of resistant starch [38], and *Bacteroides thetaiotaomicron* is the primary degrader of inulin [39, 40]. More cross-feeders are found within the Firmicutes phylum. For example, *Bifidobacterium* relies on *Bacteroides* breaking down inulin and xylan into oligosaccharides [40, 41]. Another instance of cross-feeding whereby one bacterium can utilize the end-products of another bacterium is demonstrated by the fact that about half of the butyrate-producers isolated from human faecal samples are net consumers of acetate, an end-product of other bacteria [42]. Cross-feeding requires the digesta to transit slowly to allow primary degradation and

fermentation products to become available for cross-feeding. Therefore, it is suggested that cross-feeding may be limited during small intestinal fermentation due to the shorter transit time of the digesta compared to the large intestine. This has been supported by a study finding no conversion of ^{13}C-labelled SCFAs by the ileal microbiota when infused directly into the ileal lumen of humans [43].

2.3.2. Biogeography of the human gastrointestinal tract microbiota

The GIT microbiota is highly complex and comprises an estimated 10^{13} bacterial cells [16] spread across more than a thousand species [44, 45]. These microbes include bacteria [44, 45], fungi [46, 47], viruses [48], and archaea [44, 49]. The human GIT microbiota is highly variable within different GIT regions (such as the stomach and small and large intestines) (Figure 2.2). The distinct microbiota per region is affected by factors like pH, presence of oxygen, transit time, peristaltic activity, mucus secretion, and secretion of digestive juices.

The number of bacteria in the stomach is relatively low (10^4 16S rRNA gene copies/mL chyme) due to the acidic environment, relatively short transit time (i.e., 15 min to 2 hours), and frequent peristaltic activity [50]. The predominant phyla in stomach chyme were Bacteroides, Firmicutes, Proteobacteria, and Fusobacteria. The most abundant genera were *Prevotella*, *Streptococcus,* an unclassified genus from Pasteurellaceae*, Enterococcus*, *Fusobacterium*, and *Neisseria* [50]. *Helicobacter pylori* are often found within the gastric mucosal microbiota [10]. This bacteria has been linked to gastric ulcers and a higher risk of developing diseases such as gastric cancer [51].

Throughout the small intestine, the number of bacteria increases from ≤10^3 cells/g digesta in the duodenum to 10^8 cells/g digesta in the human ileum [43, 52]. The relatively fast transit of digesta (2 to 5 hours) and the competition for nutrients with the host enzymes are the

main challenges for the small intestinal microbiota. The small intestinal microbiota is highly dynamic and can quickly adapt to the changing environment (such as substrate availability and pH) [12]. Consequently, the small intestinal microbiota is relatively unstable, less complex, and less diverse than the large intestinal microbiota [12, 20, 53]. The predominant phyla found in digesta of the small intestine were Firmicutes, Proteobacteria, and Bacteroides, and the most abundant genera are *Streptococcus, Veillonella, and Clostridium cluster XIVa* [12, 54]. The small intestinal microbiota and its function are discussed in more detail in Section 2.4.

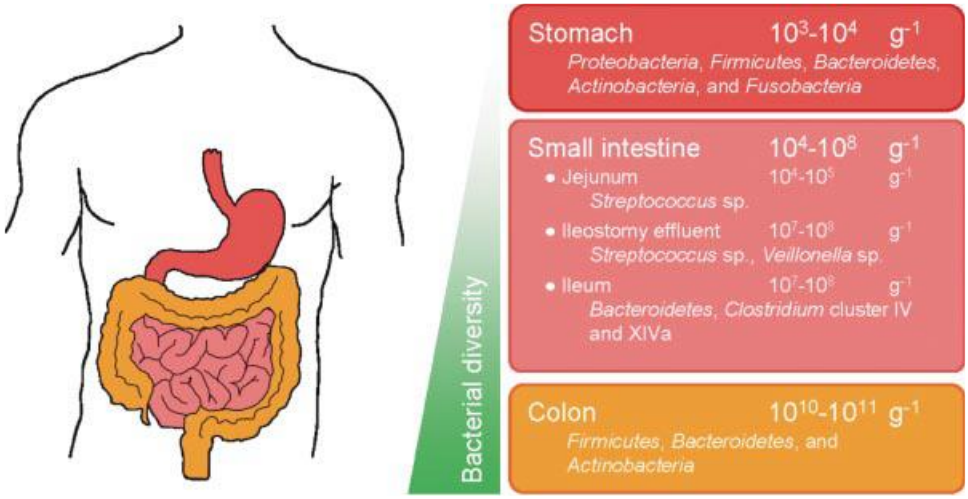

Figure 2.2: The bacterial density and predominant bacteria in various locations of the human gastrointestinal tract [55].

The human large intestine harbours 10^{10}-10^{11} bacterial cells/g digesta, including mainly obligate anaerobic bacteria [5]. The most abundant phyla in colonic digesta and faeces are Bacteroidetes and Firmicutes, followed by Proteobacteria and Actinobacteria [44, 45, 56]. The predominant genera found are *Bacteroides*, *Dorea*, *Ruminococcus*, *Eubacterium*, *Bifidobacterium*, *Clostridium,* and *Faecalibacterium* [44, 45, 56]. Even though there are

differences in phylogenetic composition, the faecal microbiota was found to be relatively comparable between individuals in terms of predicted functionality based on their genome [57]. The faecal microbiota dedicates most of its genome to carbohydrate transport and metabolism [57]. The large intestinal GIT microbiota demonstrates a high degree of functional redundancy, meaning that multiple species share similar functionality [58]. Faecal microbiota can be clustered into three so-called enterotypes, driven by the levels of *Prevotella*, *Bacteroides,* and *Ruminococcus* [59]. The different enterotypes have been found to have different fermentation capacities. For example, the *Prevotella* enterotype produced 2-3 times greater propionate during *in vitro* faecal fermentation of FOS and arabinoxylans than the *Bacteroides* enterotype [60].

The GIT microbiota described above mainly refers to the luminal microbiota, where bacteria attach themselves to the substrate in the digesta to ferment them. These bacteria often transit with the digesta and are excreted in the faeces. However, there is also a microbial community harbouring in the mucus layer, which is less transient. The mucus layer is formed by specialised epithelial cells called goblet cells in the human small and large intestines. The primary function of the mucus layer is to create a boundary between the GIT lumen and the host cells. The mucosa in the small intestine consists of only one compact layer. In contrast, the mucus layer consists of two layers in the large intestine: a loosely packed outer layer and a more densely packed inner layer. Many microbes are found in the outer layer, whereas the inner layer is virtually sterile [61]. Compared to the faecal microbiota, the mucosal microbiota has a higher abundance of bacteria of *Bacteroides* and *Allistipes* [11, 62]. Some species specialise in degrading mucins, such as *Akkermansia muciniphila* [35]. The mucosal microbiota directly influences the host's immune systems due to their proximity to the epithelial cells.

2.3.3. Fermentation end products

2.3.3.1. Short-chain fatty acids

SCFAs are volatile fatty acids with two to six carbon atoms and exist in straight or branched form (Figure 2.3). The type of SCFA and the quantity produced during GIT fermentation are affected by the substrate available. The most abundant SCFAs during faecal fermentation are acetate (C2), propionate (C3), and butyrate (C4). SCFAs are predominantly the result of carbohydrate metabolism but can also result from amino acid metabolism. SCFAs have been linked to different health benefits for the host, both locally (i.e., as an energy source of the enterocytes [2] and promote epithelial cell proliferation [63]) and systemically (i.e., play a role in glucose and lipid metabolism) [64]. It was estimated that SCFAs produced during fermentation in the large intestine supply 5 to 10% of the energy requirement for humans [65]. SCFAs also contribute to the acidification of digesta, promoting the growth of beneficial microbes and inhibiting the growth of pathogens like *Salmonella enterica* and *Escherichia coli* C1845 [66], improving mineral absorption [67], and inhibiting the conversion of primary bile acids into carcinogenic secondary bile acids [68, 69].

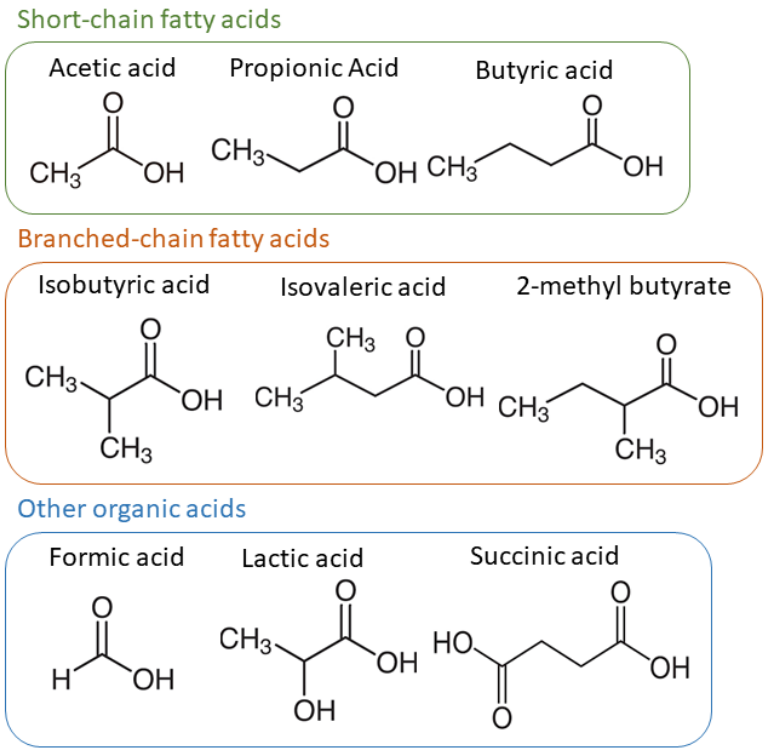

Figure 2.3: Molecular structure of the different short-chain fatty acids, branched-chain fatty acids and other organic acids produced by gastrointestinal tract microbiota.

Acetate is the predominant SCFA produced during GIT fermentation. Approximately half of the total SCFA concentration in human faeces was acetate [70]. The acetate synthesis follows (i) the oxidative decarboxylation of pyruvate or (ii) the Wool-Ljundalh pathway (i.e., acetogenesis) using hydrogen and carbon dioxide as a substrate [71, 72]. Most of the human GIT microbiota can synthesize acetate using the first pathway. Acetogenesis is more specific to acetogenic bacteria, such as *Ruminococcus* [73] and *Blautia* [74]. Approximately 36% of the acetate produced in the colon was absorbed into the bloodstream [75]. The acetate levels in the blood ranged from 70 µmol/L in the peripheral circulation to 258 µmol/L in the portal vein [23]. Via the bloodstream, the acetate is available to the muscles (e.g., cardiac and skeletal) and the brain as an energy source. Acetate acts as a substrate for cholesterol synthesis. It reduces free fatty acid concentration in blood when infused directly into the GIT

[76, 77]. Acetate can also be utilised via cross-feeding in the GIT by butyrate-producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia* [78].

GIT microbiota can synthesize propionate via three different pathways: (i) the succinate, (ii) the acrylate, or (iii) the propanediol pathways [79]. The main pathway to synthesize propionate is the succinate pathway, whereby succinate gets metabolised via decarboxylation of methylmalonyl-Coenzyme A (CoA) to propionyl-CoA. The genes encoding for enzymes that facilitate the succinate pathway can be found in the bacteria of the Bacteroidetes phylum and Negativicutes class [79]. The acrylate pathway required lactate as a substrate for propionate production and was only found in a few GIT bacteria, including *Coprococcus catus* [79]. In the propanediol pathway, deoxy sugars are metabolised into propionate. Propionaldehyde dehydrogenase, which converts propionaldehyde to propionyl-CoA, is a marker for this pathway. For example, this metabolic pathway was found in *Ruminococcus obeum*, a member of the Lachnospiraceae family [79]. Propionate production was positively correlated with *Bacteroides* and *Prevotella* during *in vitro* faecal fermentation [80]. In the GIT, propionate directly affects satiety by stimulating the production and secretion of satiety hormones, like glucagon-like peptide-1 (GLP-1), peptide YY (PYY) [81], and leptin [82]. This resulted in a greater feeling of fullness and less hunger in humans [83]. Once absorbed, propionate was transported to the liver, inhibiting fatty acid and cholesterol synthesis and reducing blood cholesterol [84].

Most butyrate-producing bacteria (~80%) in human faeces belong to the *Clostridium cluster XIVa* [42]. Half of the butyrate producers in human faeces were net acetate consumers, compared to one per cent of non-butyrate producers [42]. Acetate was converted into acetyl-coenzyme A via the butyryl coenzyme A-acetyl coenzyme A transferase pathway, which is then metabolised into butyrate [72]. This pathway can be found in most butyrate-producing GIT bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia* [78]. Butyrate is a

significant energy source for epithelial cells [2]. It has also been suggested that butyrate protects against colorectal cancer, for example, by regulating enterocyte' gene expression and differentiation properties [85, 86].

Branched-chain fatty acids (BCFAs) are products of protein fermentation. The amino acids valine, leucine, and isoleucine can be converted into the branch-chain fatty acids iso-butyrate, iso-valerate, and 2-methyl butyrate, respectively. BCFAs are found in low concentrations in the human small intestine [87]. In contrast, greater concentrations are observed in the large intestine, with increasing concentrations from the proximal to the distal colon [87, 88]. These findings suggest that proportionally more protein fermentation than carbohydrate fermentation occurs in the distal colon [88]. About 40% of the total anaerobic bacterial count found in human faeces can produce iso-butyrate [88]. A high-protein diet increased BCFA production in an *in vitro* simulated GIT model, while a diet high in dietary fibre has an inhibitory effect [80]. Colonocytes can use iso-butyrate as an energy source when butyrate concentration is low [89]. BCFAs affect lipid and glucose metabolism in human adipocytes (i.e., cells specialised in fat storage) [90].

2.3.3.2. Other organic acids

Lactate is the primary fermentation product from *Bifidobacterium, Streptococcus,* and *Lactobacillus*. These bacteria use lactate dehydrogenase to form lactate from pyruvate, an intermediate fermentation product [91]. Even though the number of lactate-producing bacteria in the human GIT microbiota is relatively high, the lactate concentration in faeces is low. The low faecal concentration of lactate was attributed to its low absorption rate [23] and the fact that lactate serves as a substrate for other GIT microbiota. Bacteria, such as *Bifidobacterium*, *Eubacterium,* and *Veillonella*, can metabolize lactate into butyrate,

propionate, and valerate [92-94]. About 20% of the total butyrate production in an *in vitro* fermentation using a human inoculum was derived from lactate [95]. Several fibres, such as inulin, FOS, and β-glucan, stimulated lactate-producing bacteria and increased lactate production during *in vitro* fermentation using human faeces [96, 97]. High colonic concentrations of lactate cause the sloughing of epithelial cells in rats [98] and are associated with inflammatory bowel disease (IBD) in humans [99, 100].

Succinate is another organic acid found in relatively low levels in the large intestine. The succinate-producing bacteria in the GIT microbiota belong mainly to the Bacteroidetes phylum [101]. The GIT epithelial cells can absorb succinate slowly due to the charge of the molecule [23]. Therefore, the primary function of succinate is as a substrate for other GIT bacteria via cross-feeding [102]. The succinate pathway is the predominant pathway to form propionate in Bacteroidetes (e.g., *Bacteroides* and *Prevotella*) and some Negativicutes [79, 102]. Like lactate, an accumulation of succinate in the GIT has been linked to inflammation and IBD [103].

2.3.3.3. Gases

Gasses are additional end products of GIT fermentation. The main gases formed are hydrogen, methane, carbon dioxide, ammonia, and hydrosulphide. Bacteria use hydrogen to dispose of reducing power that might have been generated when fermenting carbohydrates. For example, pyruvate oxidation via ferredoxin in *Clostridium* bacteria results in hydrogen and butyrate production [104]. The hydrogen produced during GIT fermentation is either excreted via the breath and flatus or utilised by methanogenic, sulphate-reducing, or acetogenic bacteria that use hydrogen as a substrate for their metabolism. In the human large intestine, two methanogenic species can utilize hydrogen. *Methanobrevibacter smithii*

uses hydrogen to reduce carbon dioxide [105], while *Methanosphaera stadtmaniae* uses hydrogen to reduce methanol, resulting in methane and water production [106]. *M. smithii* is more abundant in the human GIT than *M. stadtmaniae* [107]. Both species depend on other GIT microbes to provide the hydrogen for their metabolism. Sulphate-reducing bacteria are only found in humans who do not host any methanogenic bacteria, as these bacteria compete for hydrogen. Sulphate-reducing bacteria obtain energy by reducing sulphate to hydrogen sulphide using hydrogen [108]. Acetogenic bacteria utilize hydrogen and carbon dioxide to form acetate as their sole metabolite [71, 72]. *Ruminococcus hydrogenotrophicus* is an example of an acetogenic bacteria found in the human GIT [73]. Hydrogen cross-feeding has been reviewed in more detail by Smith *et al.*, 2018 [109].

2.3.4. Effect of diet on gastrointestinal tract microbiota and its fermentation

In addition to the intra-individual differences described above, the GIT microbiota is vastly different between individuals. These differences are affected by diet (Table 2.3), birth mode (e.g., vaginal or caesarean section delivery [110], genetics [111, 112], age [113, 114], health status [115, 116], antibiotic intake [117], geography [113] and lifestyle [118]. This part of the review focuses solely on the effect of diet as it is the main factor influencing the human GIT microbiota and provides the substrate for microbial fermentation. The GIT microbiota is affected by short-term (i.e., daily meal variations) and long-term dietary changes (i.e., changes in habitual diet). Long-term dietary patterns appear to be the strongest influencer of the GIT microbiota (Table 2.3), which, in turn, affects the functionality of the entire microbiota and the subsequential health effects (i.e., disease risk factors) for the host. It is suggested that increased microbial diversity, both in terms of the number of species and

evenness in the number of each species, allows the GIT microbiota to adapt quickly to short-term dietary changes.

Increased adherence to a Mediterranean-style diet, characterised by high consumption of olive oil, legumes, unrefined cereals, fruits, vegetables, and fish, is associated with a higher microbial diversity [119], higher abundance of fibre-degrading microbiota, such as Bacteroidetes [120], and increased SCFA concentration in faeces [121]. A plant-based or vegetarian diet is correlated with an increased abundance of the phylum Bacteroidetes and the genera *Lachnospira* and *Prevotella* [121-123]. Omnivores had a higher abundance of *Ruminococcus* [121] and Clostridium cluster XIVa bacteria, specifically *Roseburia–E. rectale* [124]. The GIT microbiota enterotypes are also correlated with long-term diets. Diets high in protein and animal fat are linked with the *Bacteroides* enterotype. In contrast, the *Prevotella* enterotype has been associated with a diet high in carbohydrates [122]. In addition to changes in microbial composition, the fermentation end products are also affected by diet. For example, faecal concentrations of acetate, propionate, and butyrate were correlated positively with fruit, vegetable, legume, and fibre consumption. In contrast, valerate and hexanoate concentrations were associated with animal protein and fat consumption [121].

Most studies on the effect of diet on GIT microbiota in humans focus on faecal samples, as sampling is non-invasive. However, this results in limited knowledge of the impact of diet on the human small intestinal microbiota (e.g., in the ileum). It has been reported that microbiota in the ileal effluent from human ileostomates fluctuates during the day, suggesting that the ileal microbiota is more affected by different meals during the day than faecal microbiota [20]. However, more research is warranted to determine the impact of diet on the human ileal microbiota.

Table 2.3: The effect of long-term diet on human faecal microbiota using next-generation sequencing. Adapted from Renall, 2020 [125][1]

Reference	Participants	Microbial analysis	Dietary assessment	Differences in microbiota related to diet
Wu *et al.*, 2011 [122]	Adults: vegetarians (*n* = 11), vegan (*n* = 1), and omnivores (*n* = 86)	Pyrosequencing and shotgun sequencing	Three 24 h recalls; FFQ	• Vegetarians showed enrichment in *Prevotella* • *Bacteroides* were associated with animal protein & saturated fat intake • *Prevotella* was associated with carbohydrate & simple sugar intake but was inversely associated with animal protein and saturated fatty acids
Kong *et al.*, 2014 [126]	French adults (*n* = 59)	SOLiD sequencing	7-day food record	• A diet characterised by a higher intake of fruit, yoghurt, soup, and vegetables and a lower intake of sweets and SSB was associated with increased richness and diversity • There was a correlation between fruit and soup intake and bacterial gene count
De Filippis *et al.*, 2016 [121]	Italian adults: vegetarian (*n* = 51), vegan (*n* = 51), and omnivores (*n* = 51)	Pyrosequencing	Self-reported diet; 7-day food record; MDS and HFD index	• Bacteroidetes was more abundant in vegetarians and vegans than in omnivores • Higher Firmicutes/Bacteroidetes ratio associated with omnivores • *Lachnospira* and *Prevotella* were associated with plant-based diets • Omnivore diet was positively associated with *Ruminococcus* • Vegans and vegetarians had higher SCFA concentrations than omnivores • Consumption of fibre, fruit, vegetables, and legumes was associated with increased SCFA concentration
Zhernakova *et al.*, 2016 [127]	Dutch adults (*n* = 1135)	Shotgun sequencing	FFQ (including 183 food items; 4-week period)	• Buttermilk was associated with higher diversity and *Leuconostoc mesenteroides* and *Lactococcus lactis* • SSB was negatively associated with alpha diversity • Coffee, tea, and wine were associated with higher alpha diversity • Red wine consumption correlated with *Faecalibacterium prausnitzii* abundance

				• Higher energy (kcal/day), full-fat milk and total carbohydrate intake were associated with lower alpha diversity • Consuming fruit and fish was associated with higher alpha diversity • Total carbohydrate intake was positively associated with *Bifidobacteria* but negatively with *Lactobacillus*, *Streptococcus* and *Roseburia* • A higher abundance of *Alistipes shahii* was associated with higher fruit intake
Menni *et al.*, 2017 [128]	Caucasian Females (*n* = 1632)	Illumina sequencing	FFQ (including 131 food items)	• Dietary fibre intake was positively associated with diversity
Bowyer *et al.*, 2018 [119]	Older European Twins (*n* = 2070)	Illumina sequencing	FFQ (including 131 food items); HEI, MDS and HFD index	• HEI and MDS were positively correlated with alpha diversity, while HFD was negatively associated with alpha diversity • HEI and MDS were negatively associated with *Ruminococcus*, *Lachnospira* and *Actinomyces*
Lin *et al.*, 2018 [129]	Two cohorts of American adults (*n* = 151)	Pyrosequencing	Semi-quantitative FFQ	• Higher dietary fibre intake was associated with a lower abundance of *Actinomyces*, *Odoribacter*, *Oscillispira*, *Eubacterium dolichum* and *Bacteroides uniformis* • Higher dietary fibre intake was associated with a higher abundance of *Clostridia: SMB53*, *Lachnospira* and *Faecalibacterium prausnitzii* • A higher intake of beans was associated with a higher abundance of *Faecalibacterium prausnitzii* and a lower abundance of *Bacteroides uniformis*
Losasso *et al.*, 2018 [123]	Italian adults: vegetarians (*n* = 32), vegans (*n* = 26), and omnivores (*n* = 43)	Illumina sequencing	Self-reported diet; Semi-quantitative FFQ (14 day-period); 24 h recall	• Vegans and vegetarians had an increased number of Bacteroidetes than omnivores

McDonald *et al.*, 2018 [130]	Adults (*n* = 11,336)	Shotgun sequencing	Self-reported diet; FFQ	• *Faecalibacterium prausnitzii* and *Oscillospira* were associated with eating > 30 types of plants • Consuming higher diversity of plants resulted in higher alpha diversity
Johnson *et al.*, 2019 [131]	American adults (*n* = 34)	Shotgun sequencing	Self-recorded dietary intake for 17 days	• Grain-based foods were positively associated with Lachnospiraceae for multiple participants • Meat-based foods were negatively associated with Bacteroidaceae for multiple participants
Partula *et al.*, 2019 [132]	French adults (*n* = 862)	Illumina sequencing	FFQ (including 19 food groups)	• Cheese was negatively correlated with *Verrucomicrobia* and *Akkermansia muciniphila* • Sweet products were negatively correlated with Proteobacteria • Fried products, soda and SSB were negatively associated with alpha diversity • Fish and raw fruit were positively associated with alpha diversity • Beta diversity was only associated with cheese, ready-cooked meals, cooked fruit, raw fruits, and fried products
Shikany *et al.*, 2019 [133]	American older men (*n* = 517)	Illumina sequencing	Semi-quantitative FFQ (including 69 foods, 1-year period)	• The “Western” pattern (processed meats, refined grains, potatoes, eggs, sweets, and salty snacks) was positively associated with *Eubacterium*, *Alistipes*, *Anaerotruncus*, *Collinsella*, *Coprobacillus*, *Desulfovibrio*, *Dorea*, and *Ruminococcus*, and negatively associated with *Coprococcus*, *Prevotella Haemophilus*, *Faecalibacterium*, *Lachnospira*, and *Paraprevotella* • The “Prudent” pattern (vegetables, fruit, nuts, fish, skinless chicken, and turkey) was positively associated with *Veillonella Faecalibacterium*, *Paraprevotella* and *Lachnospira*, and negatively associated with *Ruminococcus*, *Desulfovibrio*, *Dorea*, *Cloacibacillus*, *Collinsella*
Tang *et al.*, 2019 [134]	Healthy adults (*n* = 136)	Illumina sequencing	FFQ (including 134 food items, 1-year period)	• Sixty-one food items or nutrients were associated with at least one bacterial genus

[1] FFQ, food frequency questionnaire; HEI, Healthy Eating Index; HFD, Healthy Food diversity; MDS, Mediterranean diet score; SSB, sugar-sweetened beverages.

2.4. Evidence for small intestinal fermentation

Early research on the GIT microbiota concluded that the human small intestine is sterile [19], and no breakdown of dietary fibre occurs in the small intestine [6, 7]. Therefore, the common belief has been that GIT fermentation is limited to the large intestine due to a high number of microbes [5]. However, new microbial analysis techniques, such as next-generation sequencing, allowed for identifying many novel microbes that had not been cultured previously. This sparked further interest in microbiota in GIT locations other than the large intestine and their potential function. For example, some novel microbial strains have been isolated from the human small intestine, such as *Enterococcus sp. strain HSIEG1* [135], *Romboutsia hominis sp. strain FRIFI*^{T} [136] and *Veillonella parvula strain HSIVP1* [137]. The main challenge in researching the human small intestinal microbiota and its fermentation is the inaccessibility of the human small intestine. Small intestinal samples can be obtained via different methods, including human ileostomates [12, 20], oral/nasal intubation [12, 54], endoscopy [138, 139], and sudden death victims [9, 23]. These methods are described in more detail in Section 2.5.1.1. The predicted metabolic activity of small intestinal bacteria has been found to be different from that of large intestinal microbiota. For example, small intestinal bacteria are focused the rapid metabolism of simple sugars [12]. Therefore, small intestinal microbiota may contribute to the fermentation of dietary and non-dietary nutrients and might play an important role in supporting the host's health. The following sections give an overview of the literature to date describing different aspects of small intestinal fermentation, including the disappearance (i.e., digestibility) of dietary fibre in the upper GIT ileal (Section 2.4.1), the microbial population and its genetic potential (Section 2.4.2), and the concentration of fermentation products (Section 2.4.3). The last section (Section 2.4.4) discusses the potential function of small intestinal fermentation in humans.

2.4.1. The digestibility of dietary fibre in the upper gastrointestinal tract

Historically, the breakdown of dietary fibre was thought to only occur in the large intestine. This belief was based on several studies that reported a low or negative dietary fibre digestibility (i.e., more than 100% recovery) in human ileostomates [6, 7, 140-142]. Dietary fibres were (almost) completely fermented in the large intestine [1]. More dietary fibre was found in the ileal effluent of human ileostomates than was ingested, indicating that endogenous materials (e.g., mucins and microbial material) contribute to the total dietary fibre measured [143]. Montoya *et al.*, 2015 [144] showed that the ileal digesta of ileal-cannulated pigs contained endogenous materials that interfered with dietary fibre determination in the digesta. When the results were corrected for these endogenous materials, the ileal digestibility of the soluble fibre fraction of kiwifruit increased from 28 to 78% [144]. These findings suggest that dietary fibre digestibility might be underestimated when the results are not corrected for the non-dietary materials in the digesta.

Several *in vivo* studies have reported dietary fibre digestion (i.e., disappearance) in the human upper GIT (i.e., stomach and small intestine; Table 2.4). For example, on average, only 69% of the pectin consumed reached the terminal ileum of human ileostomates [145]. The observed ileal digestibilities ranged from 0 to 80%, depending on the type of dietary fibre and the donor. Dietary fibres are not hydrolyzed by endogenous enzymes in the human stomach and small intestine [26]. Therefore, these reported dietary fibre ileal digestibilities are likely due to microbial activity in the upper GIT. This indicates that the upper GIT microbiota plays an essential role in dietary fibre fermentation. For example, it has been demonstrated that ileal microbiota obtained from ileostomy subjects can ferment purified pectin up to 40% in 2 hours [146] and FOS up to 89% in 5 hours [21].

Table 2.4: Apparent ileal digestibility (AID) of different dietary fibres in humans, obtaining samples via ileostomy or intubation.

Reference	Dietary fibre		Dose	Collection method	*n*[1]	Fibre analysis	AID (%)	
							Range	Average
Lia *et al.*, 1996 [147]	β-glucan	As part of bread	1.2 – 13 g/day	Ileostomy	9	Monomeric sugar analysis	8 - 76	33
Sundberg *et al.*, 1996 [148]	β-glucan	As part of bread	1.1 -12.5 g/day	Ileostomy	9	Monomeric sugar analysis	13 - 64	NA
Holloway *et al.*, 1978 [149]	Cellulose	As part of a meal	6.4 -8.4 g/day	Ileostomy	6	Acid detergent extraction	0 - 31	11
Sandberg *et al.*, 1981 [150]	Cellulose	in wheat bran as part of a diet	16 g wheat bran/day	Ileostomy	9	Gravimetric, enzymatic method	0 - 26	NA
Oyama *et al.*, 2008 [151]	Cellulose	Purified	5 g in one meal	Ileal intubation	7	Monomeric sugar analysis	0 - 33	1.6
Rowan, 1989 [152]	Cellulose	As part of a meal	3.8 g/kg DM meal	Ileostomy	5	Monomeric sugar analysis	NA	8.1
Lia *et al.*, 1996 [147]	Dietary fibre[2]	As part of bread	19 - 48 g/day	Ileostomy	9	Monomeric sugar analysis	14 -34	23
Molis *et al.*, 1996 [153]	FOS	Purified	20 g/day	Ileal intubation	6	Polysaccharide profile by chromatography	0 - 40	11
Ellegård *et al.*, 1997 [142]	FOS	Purified	16 g/day	Ileostomy	8	Gravimetric, enzymatic method	0 - 37	11
van Trijp *et al.*, 2022 [43]	FOS	Purified from Chicory	5.4 g in one meal	Ileal intubation	7	Polysaccharide profile by chromatography	0 - 29	4
van Trijp *et al.*, 2022 [43]	GOS	Purified; mixture DP 2 to 6	7.1 g in one meal	Ileal intubation	7	Polysaccharide profile by chromatography	0 - 52	24
Holloway *et al.*, 1978 [149]	Hemicellulose	As part of a meal	11 -14 g/day	Ileostomy	6	Neutral detergent extraction	65 - 80	74
Sandberg *et al.*, 1981 [150]	Hemicellulose	in wheat bran as part of diet	16 g wheat bran/day	Ileostomy	9	Gravimetric, enzymatic method	0 - 21	NA

Rowan, 1989 [152]	Hemicellulose	As part of a meal	114 g/kg DM meal	Ileostomy	5	Monomeric sugar analysis	NA	45
Bach Knudsen and Hessov, 1995 [154]	Inulin	Jerusalem artichoke extract (70%)	10 - 30 g/day	Ileostomy	7	Monomeric sugar analysis	5 - 38	13
Ellegård *et al.*, 1997 [142]	Inulin	Purified	17 g/day	Ileostomy	10	Gravimetric, enzymatic method	0 - 24	12
Holloway *et al.*, 1983 [145]	Pectin	As part of a meal	4.5 - 5.6 g/day	Ileostomy	6	Uronic acid content	15 - 47	31
Sandberg *et al.*, 1983 [155]	Pectin	Purified from citrus	15 g/day	ileostomy	6	Uronic acid content	0 - 30	15
Rowan, 1989 [152]	Pectin	As part of a meal	7.5 g/kg DM meal	Ileostomy	5	Monomeric sugar analysis	NA	87
Saito *et al.*, 2005 [156]	Pectin	Purified from apple	4.1 g/day	Ileal intubation	7	Galacturonic acid content	0 - 23	12
Englyst and Cummings, 1985 [6]	Non-starch polysaccharides	In oats	6.6 g in one meal	Ileostomy	4	Monomeric sugar analysis	0 - 16	5[3]
Englyst and Cummings, 1986 [157]	Non-starch polysaccharides	In banana	2.1 g/day	Ileostomy	3	Monomeric sugar analysis	NA	1.4[3]
Sandström *et al.*, 1986 [158]	Non-starch polysaccharides	As part of a meal	14 - 16 g/day	Ileostomy	8	Monomeric sugar analysis	1 - 7	3.9
Lia *et al.*, 1996 [147]	Resistant starch	As part of bread	1.7 – 2.8 g/day	Ileostomy	9	Monomeric sugar analysis	0 - 18	1.4

[1] *n* indicated the number of participants.
[2] Dietary fibre was determined as the sum of the non-starch polysaccharides, enzyme-resistant starch, and Klason lignin.
[3] The results were corrected for endogenous galactose from mucus by correcting the galactose recovery in the effluent during the test meal day based on the fucose recovery in the effluent during the test meal day and the fucose:galactose ratio in the effluent during the fibre-free day.
AID, apparent ileal digestibility; DP, degree of polymerization; FOS, fructooligosaccharides; GOS, galactooligosaccharides; NA, not applicable

In addition to measures of ileal digestibility, it has also been shown that chemical changes in dietary fibre may occur in the upper GIT. For example, higher amounts of soluble non-starch polysaccharides (NSP) and lower amounts of insoluble NSP were found in the ileal effluent than in the diet given to humans ileostomy subjects [6], and the molecular weight of β-glucan was reduced [147]. These chemical changes in the upper GIT could also suggest microbial activity in the upper GIT. Together with the disappearance of dietary fibre by the upper GIT microbiota, these observations indicate that the small intestinal microbiota contributes to the fermentation of dietary nutrients.

2.4.2. Small intestinal microbiota

The second indication that the small intestine contributes to overall GIT fermentation is the presence of microbes in the small intestine. The small intestine imposes a challenging environment for GIT microbiota due to the short transit time (2 to 5 hours) [159], the intermitted nutrient availability, and the secretion of bile acids [160], digestive enzymes, and antimicrobial factors [161]. These conditions contribute to lower microbial density and diversity. However, it provides a niche for bacteria that adapt rapidly to environmental changes to flourish. The total number of bacteria in the small intestine varies between individuals and gradually increases from the duodenum to the ileum in human adults (Figure 2.2) [52].

Firmicutes and Proteobacteria are the two dominant phyla in the small intestine (Table 2.5). Towards the end of the small intestine (i.e., ileum), the abundance of Proteobacteria decreases, and the number of Bacteroidetes increases. A novel phylum, Saccharibacteria (formerly known as TM7), is unique to the small intestine but only represents 2% of the microbiota [162]. Most studies reported *Streptococcus* and *Veillonella* as the most abundant

genera within the small intestinal luminal microbiota. Other genera that have been found in high abundance were *Gemella* and *Prevotella* (in duodenum and jejunum), *Enterobacter* (in jejunum), *Clostridium cluster XIVa*, *Enterococcus* and *Lactobacillus* (in the ileum). An inconsistency in the taxonomic composition between studies was observed, which could be due to the different microbial analyses used. Next to that, the sampling time may have affected the taxonomic composition in the digesta, as it was found to be highly variable during the day in the ileal effluent [20, 43, 163]. The number of bacteria and the microbial composition in the ileum correlate with the meal intake, causing variability in the available nutrients [43, 163, 164]. For example, the number of *Enterobacteria* was 10^7 colony-forming units (CFU)/g digesta after a 12-hour fast, dropping to 10^3 and 10^2 CFU/g digesta at sixty and one hundred minutes, and then increasing again to 10^6 CFU/g digesta at two hundred minutes post-meal [163].

The most abundant bacteria within the mucosal microbiota in the small intestine of adult humans tended to be similar to the luminal microbiota described above [8, 11, 139, 165-171]. However, the proportions differ. The bacterial density in the mucosa obtained from the duodenum and jejunum was about 10^3-10^4 16S rRNA gene copies/g. In the terminal ileum, it is 10^5-10^6 16S rRNA gene copies/g [139]. Proteobacteria followed by Firmicutes dominated the mucosa in the duodenum and jejunum. Bacteroidetes became more abundant in the ileum. The most abundant genera in the duodenum and jejunum mucosa are *Streptococcus*, *Veillonella*, and *Prevotella*. However, more bacteria from the genera Bacteroides and Clostridium cluster XIVa are found in the ileum.

Based on predicted metabolic functionality, the small intestinal microbiota is predominantly involved in simple carbohydrate and energy metabolism [12, 54, 135, 172]. The pathways related to the sugar phosphotransferase systems and amino acid metabolism were also

enriched [12]. Many carbohydrate-active enzyme encoding genes have been found in the small intestinal microbiota, making them highly adaptive to environmental changes, such as substrate availability [12]. However, some bacteria in the small intestine cannot ferment carbohydrates. For example, *Veillonella parvula* HSIVP1 assigned only 2.4% of their genes to carbohydrate transport and metabolism [137]. Instead, more of its genes are focused on energy production and the bacterium is required to metabolize lactate, a fermentation product of *Streptococcus*, to propionate [137]. The individual *Streptococcus* strains have genes encoding for different carbohydrate-active enzymes and may focus their metabolism on different substrates. Additionally, Streptococcus is suggested to participate in inter-strain crosstalk via identical receptors found on the cell surface [173]. Combining these characteristics makes the Streptococcus community highly dynamic and adaptive, making them an ideal candidate to survive in the small intestine. Other bacteria that have features well suited for the small intestinal environment are *Bacteroides*, which can degrade bile acids and are, therefore, not affected by the high level of bile acids in the small intestine [174].

Like large intestinal microbiota, it is expected that the small intestinal microbiota is affected by diet, both short dietary interventions and long-term diet intake. However, the number of studies looking at this is limited due to the low accessibility of the human small intestine. Inter-individual variations in the ileal microbiota were observed at various times during the day, suggesting a direct effect of meal intake [20, 163]. One study described a 10-fold increase in the number of bacteria per gram of ileal effluent after a two-week intervention whereby individuals received a high-fibre diet compared to a low-fibre diet [164]. Further research is warranted to determine the interplay between small intestinal microbiota and dietary intake.

Ageing is another factor that has been studied in relation to the small intestinal microbiota. The α-diversity (i.e., species diversity within a sample) of the duodenal microbiota of adult humans decreases with chronological age [114]. However, this decrease in diversity is also correlated with an increase in medication and concomitant diseases in older individuals. The total number of bacteria in the duodenum was increased in older individuals (i.e., 66 to 80 years old), as well as the relative abundances of Proteobacteria, *Lactobacillus*, *Escherichia,* and *Klebsiella*, compared to younger individuals (i.e., 18 to 35 years old) [114]. In the case of *Klebsiella*, its relative abundance was also correlated with the number of medications. Ageing decreased the relative abundance of *Bacteroides* and an unknown genus from the family Xanthomonadaceae. These results are consistent with the effect of ageing on the large intestinal microbiota [175].

Several studies show a link between the small intestinal microbiota and the host's health status. For example, patients with celiac disease have an increased relative abundance of Proteobacteria in the duodenal mucosa than healthy adults [168]. Further, *Escherichia coli* in the ileal mucosa is associated with patients diagnosed with Crohn’s disease and can potentially initiate inflammation in the epithelial cells [176]. Due to the link between the small intestinal bacteria and several diseases, it is suggested that there might be a potential to develop strategies to target changes in the small intestinal microbiota to prevent or treat specific GIT disorders.

Table 2.5: The predominant bacterial phyla and genera identified in the digesta obtained from various parts of the small intestine in healthy adult humans and ileal effluent from human ileostomates using culture-independent methods[1]

Reference	Location	Collection Method	*n*[2]	Microbial analysis	Predominant bacteria[3]
Angelakis *et al.*, 2015 [54]	Duodenum	Oral intubation	5	Pyrosequencing and Illumina sequencing	Phyla: Firmicutes, Actinobacteria, and Proteobacteria Genera: *Streptococcus*, *Actinomyces*, *Propionibacterium*, *Granulicatella*, and *Gemella*
Zmora *et al.*, 2018 [139]	Duodenum	Endoscopy	29	Pyrosequencing	Genera: *Streptococcus*, *Prevotella*, *Bacteroides, Haemophilus*, and *Veillonella*
Mailhe *et al.*, 2018 [177]	Duodenum	Endoscopy	6	Culturomics and Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Bacteroidetes
Seekatz *et al.*, 2019 [178]	Duodenum	Intubation	9	Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Bacteroidetes Genera: *Streptococcus*, *Veillonella*, *Gemella*, an unclassified genus from the Pasteurellaceae family and *Fusobacterium*
Leite *et al.*, 2021 [114]	Duodenum	Intubation	251	Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Actinobacteria Genera: *Streptococcus, Veillonella*, *Prevotella*, *Rothia* and *Escherichia*
Maeda *et al.*, 2022 [179]	Duodenum	Endoscopy	34	Illumina sequencing	Phyla: Firmicutes, Bacteroidetes, and Proteobacteria Genera: *Streptococcus, Prevotella*, *Veillonella*, *Fusobacterium*, and *Neisseria*
Zheng *et al.*, 2022 [180]	Duodenum	Endoscopy	5	Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Fusobacteria Genera: *Veillonella*, *Fusobacterium*, *Streptococcus, Neisseria*, and *Prevotella*

Hayashi *et al.*, 2005 [9]	Jejunum	Sudden death victims	3	T-RFLP analysis	Phyla: Firmicutes and Proteobacteria Genera: *Lactobacillus, Streptococcus*, *Enterobacter*, and *Klebsiella*
Zoetendal *et al.*, 2012 [12]	Jejunum	Nasal intubation	1	HITChip	Phyla: Firmicutes and Proteobacteria Genera: *Streptococcus, Veillonella,* and *Clostridium* cluster XIVa
Sundin *et al.*, 2017 [138]	Jejunum	Endoscopy	20	Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Bacteroidetes Genera: *Streptococcus*, *Prevotella*, *Veillonella*, *Escherichia*, and *Fusobacterium*
Zmora *et al.*, 2018 [139]	Jejunum	Endoscopy	29	Pyrosequencing	Genera: *Streptococcus*, *Prevotella*, *Bacteroides, Haemophilus*, and *Veillonella*
Seekatz *et al.*, 2019 [178]	Jejunum	Intubation	6	Illumina sequencing	Phyla: Firmicutes and Proteobacteria Genera: *Streptococcus*, *Gemella*, *Veillonella*, an unclassified genus from the Pasteurellaceae family and an unclassified genus from the Enterobacteriaceae family
Shin *et al.*, 2019 [181]	Jejunum	Endoscopy	12	Illumina sequencing	Genera: *Streptococcus*, *Veillonella*, and *Actinomyces*
Leite *et al.*, 2020 [162]	Jejunum	Intubation	23	Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Actinobacteria Families: Steptococcaceae, Enterobacteriaceae, Veillonellaceae, Micrococcaceae, and Pseudomonadaceae
Hayashi *et al.*, 2005 [9]	Ileum	Sudden death victims	3	T-RFLP analysis	Phyla: Proteobacteria and Firmicutes Genera: *Bacillus*, Lactobacillus, *Streptococcus*, *Actinobacillus*, and *Enterococcus*
Booijink *et al.*, 2010 [20]	Ileum	Ileostomy	4	DGGE	Phylum: Firmicutes Genera: *Streptococcus*, *Veillonella*, *Enterococcus*, and *Clostridium* cluster I

Zoetendal *et al.*, 2012 [12]	Ileum	Nasal intubation	3	HITChip	Phyla: Bacteroidetes, Firmicutes, and Proteobacteria Genus: *Clostridium* cluster XIVa
Zoetendal *et al.*, 2012 [12]	Ileum	Ileostomy	5	HITChip	Phylum: Firmicutes Genus *Bacillus Clostridium* cluster XIVa, XI, I, and IV
Leimena *et al.*, 2013 [182]	Ileum	Ileostomy	2	Pyrosequencing and Illumina sequencing	Phyla: Firmicutes and Proteobacteria Genera: *Streptococcus*, *Clostridium*, *Veillonella*, *Haemophilus*, and *Turicibacter*
van den Bogert *et al.*, 2013 [183]	Ileum	Ileostomy	4	Pyrosequencing	Phyla: Firmicutes and Bacteroidetes Genera: *Streptococcus, Enterococcus, Veillonella, Bacteroides,* and *Lactobacillus*
Mailhe *et al.*, 2018 [177]	Ileum	Endoscopy	6	Culturomics and Illumina sequencing	Phyla: Firmicutes, Proteobacteria, and Bacteroidetes
Zmora *et al.*, 2018 [139]	Ileum	Endoscopy	29	Pyrosequencing	Genera: *Bacteroides*, a genus of the Lachnospiraceae family, a genus of the Enterobacteriaceae family, *Faecalibacterium,* and *Streptococcus*
Ruigrok *et al.*, 2021 [172]	Ileum	Ileostomy	57	Shotgun sequencing	Phyla: Firmicutes and Proteobacteria Genera: *Streptococcus*, *Escherichia*, *Blautia*, *Peptostreptococcaceae noname*, *Clostridium*, *Lactobacillus*, and *Veillonella*
Nana *et al.*, 2021 [184]	Ileum	Ileostomy	11	Illumina sequencing	Genera: *Escherichia/Shigella*, *Turicibacter*, *Clostridium*, *Bacteroides*, and *Haemophilus*
van Trijp *et al.*, 2022 [43]	Ileum	Intubation	6	Illumina sequencing	Genera: *Haemophilus*, *Clostridium sensu stricto 1*, *Streptococcus*, a genus of the Enterobacteriaceae family, and *Veillonella*

[1] HITChip, Human Intestinal Tract Chip; T-RFLP, Terminal Restriction Fragment Length Polymorphism; DGGE, Denaturing gradient gel electrophoresis.
[2] *n* indicated the number of participants. If the study included both patients and healthy controls, only the results of the healthy controls were included in this table.
[3] The predominant bacteria are listed in the most abundant phyla (up to three), families, or genera (up to five).

2.4.2.1. Comparing the microbiota from the small and large intestine

The small intestinal microbiota differs from the large intestinal microbiota in terms of the number of bacteria and the taxonomic diversity. For example, with 10^{10}-10^{11} cells/g digesta [5], the number of bacteria in the large intestine is about a hundred to a thousand times greater than that of the ileum [52]. The most abundant phyla found in the luminal microbiota are similar between the small and large intestines, namely Firmicutes, Proteobacteria, and Bacteroidetes. However, the relative abundance of these phyla differs. For example, a higher relative abundance of Firmicutes is found in the large intestine. One phylum, Saccharibacteria, was unique to the small intestine that has not been found in the large intestine [162]. The genera within the most abundant phyla also differed between small and large intestinal microbiota. For example, a higher relative abundance of *Veillonella*- and *Streptococcus* were found in the small intestine lumen than in the large intestine [8, 9, 20, 162, 172]. On the other hand, *Bacteroides* and *Clostridium* are found in higher relative abundance in the large intestine than in the duodenum and jejunum [8, 9, 20, 172]. It is found that the microbiota in the duodenum and jejunum resemble more the microbiota in other upper GIT locations (i.e., stomach and oesophagus) [139, 171]. At the same time, the ileal microbiota was more similar to the colonic and faecal microbiota in terms of the most abundant genera. Of the three enterotypes described for the faecal microbiota only one, namely the *Prevotella* enterotype has been identified in the small intestine (i.e., jejunal mucosa) [169]. The other enterotypes have not been found in the small intestine, which is expected since *Ruminococcus* and *Bacteroides* do not belong to the most abundant genera in the small intestine. A lower α-diversity was found in the ileal effluent of human ileostomates than the α-diversity in the faeces of human adults [20, 172]. Like the large intestinal microbiota, the small intestinal microbiota is unique per individual. However, the

small intestinal microbiota is less stable and fluctuates more than the large intestinal microbiota [20].

More bacteria were found in the human ileal mucosa (i.e., 10^9 16s rRNA gene copies/mg tissue) than in the colon (i.e., 10^7 16s rRNA gene copies/mg tissue) [165]. The mucosal microbiota composition is also different in the small and large intestines. For example, a greater relative abundance of *Bifidobacterium* is found in human ileal mucosa, while *Lactobacillus*, *Eubacterium*, and *Faecalibacterium* are more dominant in colonic mucosa [165]. The α-diversity was higher in mucosal samples from the human large intestine compared to the jejunum but not the ileum [8]. The β-diversity also showed that the ileal mucosal microbiota was similar to the mucosa in the colon [167].

The predicted functionality of the small intestinal microbiota was found to be different from the faecal microbiota. The metabolic pathways enriched in the small intestinal microbiota compared to the faecal microbiota were related to carbohydrate metabolism, energy metabolism, amino acid biosynthesis, and biotin biosynthesis [12, 54, 172]. On the other hand, some pathways, such as the urea cycle, nitrogen fixation, and methanogenesis, were more enriched in the large intestinal microbiota [12]. While the small intestinal microbiota appears to have adapted to a quickly changing environment, the large intestinal microbiota has evolved to survive in an environment low in simple sugars. It has optimised its metabolism to long-chain carbohydrates. These differences in predicted metabolic activity are based on differences in the genome between faecal and ileal microbiota and do not directly represent functionality. Nevertheless, it is suggested that the small intestinal microbiota has a different GIT function and fermentation capacity than the large intestinal microbiota.

Currently, most GIT microbiota research is focused on faecal samples due to the non-invasive nature of the sampling. However, the differences in microbial composition, diversity, and predicted metabolic functionality between the small and large intestinal microbiota in humans demonstrate that the faecal microbiota does not reflect the microbiota present in the small intestine and, therefore, may provide an incomplete picture of GIT fermentation and including other GIT locations is important to better understand GIT fermentation. It is proposed to study the small intestinal microbiota directly to better understand the GIT microbiota, its fermentation, and its potential health effect on the host.

2.4.3. Fermentation products in the small intestine

Another indicator of small intestinal fermentation is the presence of bacterial metabolites. Several studies have reported the presence of fermentation products in the small intestine. For example, 34.5 mmol total organic acids/kg ileal digesta were found in sudden death victims [23] and 25 mmol total SCFAs/L ileal effluent [185]. The main organic acids found in the small intestine were lactate (38%), succinate (23%), and acetate (22%) [23]. However, in the faeces of healthy adult humans, the total SCFA concentration was four times higher than in ileal effluent with higher amounts of butyrate and propionate [185]. Low to non-detectible amounts of iso-valerate and iso-butyrate are found in ileal digesta [23]. This was expected as ileal microbiota was not involved in protein fermentation, based on the ileal digestibility of protein in human ileostomates after an antibiotic treatment being similar to before the antibiotic treatment [186, 187]. Like in the large intestine, organic acid production can be affected by diet. Human ileostomates receiving a high-fibre diet containing rye bread had an increased concentration of SCFAs and lactate in their ileal effluent compared to those receiving a low-fibre diet containing wheat bread [188].

It should be noted that SCFA concentrations in ileal digesta or effluent only represent a single time point and do not reflect the total production during small intestinal fermentation. *In vitro* fermentation may better represent the total SCFAs produced during fermentation, as it is not affected by absorption by the host. Up to 120 mM SCFAs, mainly acetate, were produced during 5 hours of *in vitro* fermentation of purified dietary fibre substrates with ileal effluent [21, 146]. The type and amount of organic acid produced depended on the dietary fibre substrate and the ileal effluent donor [21].

2.4.4. Potential health effects of small intestinal fermentation

The following paragraphs discuss the health effects that may be attributed specifically to small intestinal fermentation (i.e., its function in the human GIT) and two disease states with either reduced or high microbial activity in the small intestine.

2.4.4.1. Positive health effects ascribed to small intestinal fermentation

Firstly, small intestinal microbiota helps to break down nutrients and recover energy. For example, germ-free mice have reduced lipid digestion and absorption in the duodenum and jejunum [189]. Introducing jejunal microbiota into the germ-free mice increased lipid absorption, which was linked to the bacteria from the Clostridiaceae family [189]. Similar results are expected in humans as ileal microbiota from human ileostomates have a higher predicted activity of four metabolic pathways related to fatty acid and lipid metabolism than faecal microbiota from adult humans [54, 172]. Research on different animal species found that species with a high-fibre diet benefit more from small intestinal fermentation to retrieve energy from nutrients resistant to the host digestive enzymes [190]. Therefore, it is expected

that humans that consume a high-fibre diet would benefit more from small intestinal fermentation.

Secondly, small intestinal microbiota may play an important role in converting specific dietary components and improving their bioavailability. For example, ileal microbiota from human ileostomates was found to metabolize polyphenols from green tea during *in vitro* fermentation, providing smaller bioactive compounds that could be absorbed by the host and serve as antioxidants [191]. There have also been reports that small intestinal microbiota, especially in the duodenum, can support gluten digestion in human adults and patients with celiac disease [192, 193]. Some of these bacteria (e.g., *Lactobacillus*) can metabolise the immunogenic 33-mer peptide as observed *in vitro* [194] and in a mouse model [192]. The end products of bacterial fermentation of gluten could be harmless or harmful [192]. More research is required to understand the role of specific small intestinal bacteria and their potential to reduce the immunogenicity of gluten.

Thirdly, the small intestinal microbiota is enriched in genes related to the synthesis of various vitamins compared to the faecal microbiota [12, 172]. For example, all small-intestinal derived *Streptococcus* have a genome enriched with genes to synthesize folate (vitamin B9) and pyridoxal-5-phosphate (vitamin B6) [173]. In addition, *Pseudomonas* and *Klebsiella*, isolated from human small intestinal digesta, produce significant amounts of vitamin B12 (cyanocobalamin) [195]. The host can absorb the vitamins directly, making the small intestinal microbiota an important supplier of these essential vitamins.

The small intestine is the GIT location where more interactions between the intestinal microbiota and host immune cells happen due to the high density of immune cells (i.e., Peyer's patches) and a thinner mucus layer [196]. Several bacteria (e.g., *Lactobacillus* and *Bacteroides thetaiotaomicron*) in the ileum have been found to regulate gene expression in

epithelial cells *in vivo* [197] and in a mouse model [198, 199]. There are also specific bacteria, namely segmented filamentous bacteria (SFB), found in the small intestine [200] that can directly affect the immune system, for example, by inducing cytokine production [201, 202]. SFBs anchor themselves in the epithelium layer near the Peyer's patches in the ileum [202]. Therefore, it is suggested that the ileal microbiota is an important player in driving the innate and adaptive immune system of the host.

Lastly, the blood level of acetate of human ileostomates is about 44% of that of healthy adult humans, implying that small intestinal fermentation contributes to systemic health effects via the absorption of fermentation products [203]. Multiple studies have linked end products of GIT fermentation with appetite control in human adults [204]. It is expected that this mechanism is through the free fatty acid receptors that are found in the small intestine. These receptors produce satiety hormones (i.e., GLP-1, ghrelin, and PYY) upon binding with fermentation products such as acetate. Therefore, it is hypothesised that small intestinal fermentation reduces appetite. The free fatty acid receptors in the ileum can also trigger a negative feedback pathway to lower glucose production in the liver upon binding with propionate in a rat model [205]. Next to inducing satiety hormone production, organic acids also stimulate ileal motility in humans [206, 207]. Therefore, small intestinal fermentation may promote the movement of digesta along the GIT.

2.4.4.2. Adverse health effects related to the small intestinal microbiota and fermentation

Two disease states could provide insights into the function of small intestinal fermentation and the effects of dysbiosis when there are either too few or too many bacteria in the small intestine. Short bowel syndrome (SBS) is a state whereby part of the small intestine has

been removed due to a GIT resection. Patients with SBS have reduced microbial activity in the small intestine. A higher abundance of *Lactobacillus,* a member of the typical small intestinal microbiota, is found in the faeces of patients with short bowel syndrome [208, 209]. In comparison, the relative abundance of *Clostridium* and *Bacteroidetes*, typical large intestinal bacteria, is lower in the faeces of patients with SBS than in healthy individuals [208, 209]. Similar results were obtained in patients who received a duodenal-jejunal bypass excluding about 60 cm of the small intestine [210]. These differences in the microbiota suggest that when the small intestine is short, the function that the *Lactobacillus* and other bacteria would perform in the small intestine is shifted to the large intestine. These results strengthen the suggestion that the microbiota in the small intestine performs an important role. However, it is unknown whether the GIT fermentation and the production of metabolites differ between patients with SBS and healthy humans. In addition, the malabsorption of nutrients due to the short bowel may also affect the large intestinal microbiota in patients with SBS.

Secondly, small intestinal bacterial overgrowth (SIBO) is a disease whereby microbial activity in the small intestine is above normal causing adverse health effects such as bloating. In SIBO, the duodenum, jejunum, or proximal ileum is colonised by many bacteria, usually classified as typical large intestinal bacteria [211]. A diagnosis of SIBO is the presence of $\geq 10^3$ CFU/mL of duodenal digesta [212] or $\geq 10^6$ CFU/mL of jejunal digesta [211]. The overload of bacteria can interfere with the digestion and absorption of nutrients. For example, SIBO is associated with the loss of brush-border enzymes due to mucosal injury and bacterial fermentation of sorbitol, fructose, and lactose, resulting in the malabsorption of carbohydrates [213]. An increased relative abundance of Enterobacteriaceae, *Escherichia-Shigella*, and *Clostridium perfringens* is found in the duodenum patients with SIBO compared to non-SIBO individuals [214, 215]. In addition, duodenal digesta of patients

with SIBO had a decreased abundance of Firmicutes and lower α-diversity than non-SIBO individuals [215]. The duodenal microbiota of SIBO subjects has a different predicted metabolic function (based on PICRUSt analysis) than that in non-SIBO individuals. For example, pathways reflective of oxidative stress and simple sugar metabolism increased in SIBO subjects [216]. A higher prevalence of SIBO has been observed in patients with several health conditions, such as non-alcoholic fatty liver disease, functional dyspepsia [217], inflammatory bowel disease [218], and Alzheimer's disease [219], compared to healthy controls. These two disease states demonstrate that there should be a balance between enough small intestinal microbes to provide positive health benefits but not too many, leading to adverse effects.

2.5. Fermentation models

The common approaches to researching human GIT microbiota and their fermentation are based on human faecal material. However, the faecal microbiota does not resemble the small intestinal microbiota. The inaccessibility of the human small intestine makes it challenging to research the small intestinal microbiota and its fermentation. The following sections discuss (i) several techniques to obtain small intestinal samples from human adults, (ii) animal models that could be used instead of humans, and (iii) *in vitro* models designed to mimic the GIT fermentation and their suitability to study small intestinal fermentation.

2.5.1. In vivo *models*

2.5.1.1. Humans

Sampling directly from the small intestine of healthy human adults to investigate human small intestinal fermentation is believed to be ideal. The following section discusses several methods to obtain small intestinal samples, including the benefits and challenges.

2.5.1.1.1. Sudden death victims

Small intestinal samples can be obtained from sudden death victims [9, 23]. After obtaining consent from their relatives, samples can be taken during autopsy from different GIT regions within several hours of death. A concern with this sampling method is that post-mortem changes may affect the samples, such as the continuation of fermentation and shedding of epithelial cells. However, it has been shown in pigs that no distinguishable differences happened in the GIT microbiota, pH, and SCFA concentration during four hours post-mortem [23]. It is unclear whether post-mortem changes affect the fermentative capacity of the GIT microbiota. Sampling from sudden death victims allows the identification of microbial communities and metabolites in multiple GIT regions in the same subject. However, there is no option for dietary interventions.

2.5.1.1.2. Surgery

Another option to obtain samples from the small intestine directly is during surgery, for example, by needle aspiration [19, 138], swabbing the luminal wall [220], or mucosal biopsy [8, 11, 165, 170, 171]. Surgical sampling avoids contamination; therefore, the samples obtained accurately represent the small intestinal microbiota. However, surgery is invasive,

and it is nearly impossible to get samples from healthy controls. Also, surgical sampling often requires GIT preparation, such as fasting, cleansing, and antibiotic treatment, before surgery which could disrupt the microbiota [221]. Some studies have taken samples from patients undergoing emergency surgery to avoid pre-surgery GIT preparation [19, 165]. Obtaining samples during surgery allows for investigating the microbiota. However, the sample is often too small to perform a comprehensive analysis to assess fermentation patterns, and dietary interventions may not be possible.

2.4.1.1.3. Intubation

Intubation is another method used to obtain luminal content from the small intestine of healthy human adults. A tube is inserted via the nasal or oral cavity, swallowed, and guided to the desired location. Human subjects are often fasted overnight before positioning the tube in the small intestine, which requires gastroenterologist supervision. The position of the tube can be determined using fluoroscopy. Small aspirates (~ 1 mL) can be collected via the tube. The various intubation techniques available are discussed in more detail by van Trijp *et al.*, 2021 [222]. Intubation has been used successfully to determine the ileal digestibility of dietary fibre [151, 156], small intestinal microbiota [12, 54, 162, 178], and SCFA concentrations [206]. However, there are concerns about whether the small digesta samples are representative. Also, if the intestinal fluid is viscous, it can cause blockage in the tubing making the procedure challenging and time-consuming. It is suggested to use this methodology only for highly digestible diets. In addition, intubation is an invasive procedure, has a high drop-out rate, and is unsuitable for repeat sampling. Therefore, this methodology is not widely used.

2.4.1.1.4. Ileostomy subjects

Human ileostomates have been used to sample digesta from the end of the small intestine (i.e., ileum) [12, 20, 53]. Human ileostomates underwent surgery where the colon is completely removed (i.e., permanent ileostomy) or bypassed to allow healing (i.e., temporary ileostomy) due to conditions such as colorectal cancer, ulcerative colitis, or Crohn's disease. The terminal ileum is connected to an abdominal stoma that allows the excretion of ileal effluent. The collection of ileal effluent is non-invasive to the human ileostomates, inexpensive, and allows repeated sample collection and dietary interventions.

It has been suggested that human ileostomates do not have an ileal microbiota representative of that in an intact adult human due to potential exposure to oxygen and changes in the anatomical structure after surgery [53]. Even though the presence or absence of oxygen after an ileostomy has not been confirmed, the presence of strictly anaerobic bacteria (such as *Ruminococcus gnavus*) in ileal effluent suggests that oxygen has a limited effect on the microbiota in ileal effluent [12, 20]. Several studies compared the microbiota in ileal effluent with ileal samples obtained from healthy human adults via intubation [12, 223]. Gorbach *et al.*, 1967 [223] found that the number of bacteria was greater in the ileal effluent. However, the distribution of aerobic and anaerobic species was similar. A recent study found that the microbiota in the ileal effluent more closely resembled the microbiota of the proximal small intestine (i.e., jejunum) than that of the ileum of healthy adult humans [12]. A possible explanation for this observation could be the removal of the ileocaecal valve, which reduces the transit time of the ileum [224], and the absence of colonic reflux [12].

Human ileostomates are a valuable model for studying ileal fermentation despite the abovementioned limitations. They provide sufficient samples to perform experiments testing

fermentation behaviours [21]. This model provides opportunities to explore the dynamics within the small intestinal community over time or through dietary interventions. However, recruiting enough participants is difficult for routine tests.

2.4.1.1.4. Ingestible devices

Ingestible devices are another option to investigate the GIT microbiota (including small intestine microbiota) and its fermentation. These small devices can be swallowed and measure fermentation products [225, 226] or take intestinal samples [227-234]. The first group of devices is programmed to sense different gasses, such as methane, oxygen, hydrogen, and carbon dioxide. It allows real-time analysis of gas profiles in the human GIT after food intake [225, 226]. The location of the capsule within the GIT is determined by the oxygen-equivalent concentration [225].

Several ingestible devices have been developed to sample luminal contents [228, 231, 232] or mucosa [234] along the human GIT and avoid contamination using enclosed capsules. The opening and closing of the capsules are regulated by negative pressure [228], pH changes in the GIT [232], or wireless communication [231, 234]. Other capsules can obtain mucosal biopsies from the GIT [227, 229, 230]. The location of the capsule is tracked using fluoroscopy [227]. Once it is in the desired location, the tissue is drawn into the capsule using suction or pressing against the intestinal wall, after which a cutting device is used to obtain the biopsy. Cummins, 2021 [231] have conducted a more comprehensive review of these different ingestible devices.

Ingestible devices allow for accurate sample collection in the GIT and collect sufficient samples to enable studying the luminal or mucosa microbiota at a genetic level. However, these techniques are expensive and require advanced technical knowledge. Importantly, not

enough sample can be obtained to perform *in vitro* fermentation experiments to determine fermentative capacity and metabolite production.

2.5.1.2. Animal models

Human subjects are the gold standard when researching the GIT microbiota and fermentation. However, human experiments have disadvantages, including cost, ethical considerations, safety concerns, and dietary compliance. It can also be challenging and invasive to sample the human small intestine. Therefore, *in vivo* animal models have been used to study the human small intestine microbiota and its fermentation. An additional advantage of using animals is that it reduces the individual variation caused by genetic and environmental factors. Several animals have been used previously as models for the human adult to study the GIT microbiota and its fermentation, including mice, rats, dogs, and primates. Even though dogs and primates have similar intestinal fermentation and diet (i.e., omnivorous) as humans [235, 236], these animals are hardly used due to the higher cost, emotional attachment, and animal welfare issues.

Rodents have been more frequently used as animal models for human adults since they are cost-effective. However, some physiological and metabolic differences between rodents and adult humans have been found. For example, rodents are granivore animals, nocturnal eaters, selective nibblers, and practice coprophagy. In addition, rodents have a faster transit of the digesta, resulting in a reduced ability for potential intestinal fermentation. Like in adult humans, the main phyla in the faecal microbiota of rodents are Firmicutes and Bacteroides [237]. However, the relative abundance of important bacterial genera differs between rodents and humans, for example, *Lactobacillus* and *Bifidobacterium* [238-240]. These

differences and the fact that only a small digesta sample can be obtained from rodents make them unsuitable for comprehensive analysis to study small intestinal fermentation.

The growing pig has also been used as an animal model for the adult human in nutritional studies, as the GIT physiology of the stomach and small intestine is comparable with that of humans [14, 241-243], including similar transit times of the digesta [14], digestion activities, and absorption [244]. Pigs are monogastric and omnivorous meal eaters like humans. Moreover, the nutrient requirements of the pig are similar to the recommended daily nutrient allowances for adult humans [14]. Therefore, pigs have also been proposed as an adequate model for studying GIT microbiota and its fermentation [245, 246].

The main phyla in the GIT microbiota of pigs are Firmicutes and Bacteroidetes phyla [14, 247, 248], which is similar to that of adult humans [44, 45]. However, differences have been found at the genus level based on independent studies. For example, *Prevotella* is the most abundant genus in the porcine faecal microbiota [249]. In contrast, the most abundant genus in human faeces is *Bacteroides* [44, 45]. The abundance of *Lactobacillus* and *Streptococcus* is greater in the porcine faeces [247, 249, 250], while *Bifidobacterium* and *Bacteroides* are increased in the human faeces [44, 45]. These differences in the GIT microbiota between pigs and humans have been associated with differences in diet [251] and environmental hygiene [252]. In addition, the fermentative capacity of the microbiota may also differ between the species. For example, when humans and pigs were fed a similar diet, cellulose and pectin ileal digestibility was higher in pigs than in humans [152]. However, the ileal digestibility of hemicellulose was similar between the species [152]. More research is warranted to compare the functionality of the human and pig microbiota.

An advantage of pigs over rodents is the larger size of the GIT (i.e., larger sample size), allowing for a more comprehensive analysis of the GIT microbiota and its fermentation.

However, pigs are not as cost-effective as rodents since they need more feed, animal care, and larger housing facilities. Samples from the small intestine of pigs can be obtained after euthanasia (in addition to samples from other GIT locations) or via ileal- or jejunal cannulation of a GIT location, which allows for repeat sampling. Despite the limitations described above, the pigs are suggested to be a good model for human small intestinal fermentation.

2.4.1.2.1 Human-flora-associated animals

As discussed above, the main criticism of using animal models to study human microbiota is that the microbiota can differ between species. Human-flora-associated (HFA) animals reduce the inter-species differences in GIT microbiota. Human faecal microbiota was transplanted into gnotobiotic or germ-free animals (e.g., pigs and mice) within the first 12 hours after birth and for several days thereafter to obtain these HFA animals [253-258]. HFA animals have a microbiota that more closely resembles the microbiota of the human donor, both in taxonomic composition and metabolic function *[257]*. The human adult microbiota was more successful in colonizing the GIT of HFA pigs than HFA mice [258]. Some specific genera present in the microbiota of the human donor, namely Bifidobacterium, Lactobacillus, and Clostridium, were unable to colonize in the murine GIT [253, 254]. In contrast, these bacteria could colonize the pig GIT [255, 256]. Therefore, using HFA pigs as an animal model for human GIT microbiota is preferred over HFA mice. The HFA animal model can increase the reproducibility of experiments if a standardised stock of microbiota is preserved at -80°C. A standardised microbiota stock allows inoculating multiple batches of animals with the same microbiota.

However, inoculating animals with human faecal microbiota has not always been successful. Even though the human donor appears healthy (i.e., without clinical symptoms), transplanting its microbiota into pigs may introduce opportunistic pathogens that could be fatal for the pig [259]. This finding demonstrates the importance of screening the microbiota of the human donor for specific pathogens before inoculating the pigs.

Despite its limitations, the HFA animals, especially pigs, provide a valuable tool to study how environmental and generical factors affect the GIT microbiota and its metabolism in conditions like the human GIT. HFA animals are considered the preferred animal model over conventional animals. However, the current research that included HFA animals has been limited to the faecal microbiota. The effect of the human faecal inoculum on the small intestinal microbiota of HFA animals is unknown.

2.5.2. In vitro *models*

Even though *in vivo* models are preferred to investigate the GIT microbiota and its fermentation, it is not always possible to use *in vivo* models. Several fermentation outcomes (e.g., metabolite production) cannot be measured accurately due to processes in the GIT, such as absorption. Therefore, various *in vitro* models have been developed to mimic human GIT fermentation.

2.5.2.1. Static batch fermentation models

In vitro static batch fermentation models are the simplest and most frequently used models to study the fermentation behaviours of human faecal microbiota [30, 260, 261]. The substrate (e.g., dietary fibre) and microbiota (i.e., inoculum) are added at time zero to the

reaction vessel, after which the fermentation is allowed to proceed under a controlled environment (e.g., fixed temperature and anaerobic conditions). A standardised methodology to simulate large intestinal fermentation uses 100 mg DM substrate of fibre substrate and 10 mL of inoculum. The inoculum is prepared by diluting the human faecal matter with a phosphate buffer in a 1:4 (w:v) ratio. Both the preparation of the inoculum and the fermentation are done under anaerobic conditions at 37°C [260]. This approach has been used to evaluate the ability of individual microorganisms, multiple microbial strains, or whole microbial communities to metabolize different substrates. Short-chain fatty acid production and the disappearance of the substrate are often measured at the end of the fermentation process. However, several methodologies, such as the cumulative gas production technique [262], include repeat sampling during the fermentation to understand the fermentation kinetics. Batch fermentations are easy, cheap, standardised, and allow many samples to be studied simultaneously. It is often used in pre-screening approaches, allowing for a relative comparison between many different substrates.

These *in vitro* batch systems are simplified models of the *in vivo* fermentation in humans since they do not reproduce the dynamic processes that occur during human digestion and fermentation, such as the flow of digesta, addition of digestive juices (i.e., bile acids), and absorption of nutrients. On the other hand, an advantage of *in vitro* batch fermentation over *in vivo* models is that the latter cannot accurately predict the production of microbial metabolites due to absorption.

Currently, standardised *in vitro* static batch models are available that mimic digestion in the human small intestine [263]. However, these models do not include any small intestinal microbiota. Some studies have used ileal digesta from cannulated dogs and pigs to prepare an inoculum to perform *in vitro* small intestinal fermentation [264-267]. More recently, an inoculum prepared from ileal effluent from human ileostomates was tested for its ability to

ferment different fibre sources [21, 146]. However, in the latter studies, samples were fermented for over 5 hours, which might not be an accurate physiological representation of human ileal fermentation time as the transit time of digesta in the ileum is around 2 hours. Next to that, a protocol was developed to cultivate a simplified microbial community to represent the ileal microbial community, consisting of five different bacteria; *Bacteroides thetaiotaomicron*, *Bifidobacterium longum*, *Lactobacillus plantarum*, *Streptococcus salivarius* and *Escherichia coli* [268]. Using this simplified ileal inoculum could increase the reproducibility of *in vitro* ileal fermentation experiments. However, whether this simplified ileal inoculum has fermentation behaviours similar to the ileal microbiota found in humans is unknown. Therefore, more research is warranted on whether static *in vitro* models can accurately represent the *in vivo* small intestinal fermentation.

2.5.2.2. Continuous dynamic models

Even though *in vitro* static batch experiments provide a straightforward and flexible tool to investigate the GIT microbiota and its fermentation, they do not fully represent the complex processes in the GIT *in vivo*. Therefore, continuous and dynamic *in vitro* systems have been developed to better simulate the environmental condition in the gut, such as pH changes and passage of digesta. These continuous dynamic models allow an in-depth study of the GIT microbiota and its fermentation behaviours.

Several *in vitro* continuous dynamic systems have been developed to simulate the large intestine, including the MacFarlane and Gibson model [269, 270], the TNO intestinal Model of the colon (TIM-2) [271], and the EnteroMix Colon Simulator [272]. These are multi-compartment models in which each compartment represents a different section of the large intestine (i.e., ascending, transverse, and distal colon). Another group of *in vitro* dynamic

fermentation models combines the colonic model described above with two additional compartments to simulate the stomach and small intestinal digestion. These models include the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [273], the TNO gastric–small intestinal model (TIM-1) connected to the TIM-2 [271], and the Simulator Gastro-Intestinal [274]. All these models operate at 37°C under anaerobic conditions, but the pH is regulated differently in each compartment to represent the *in vivo* conditions. Human adult faecal microbiota is used to inoculate these models. It takes about two weeks for the microbiota to stabilize [273, 275]. The *in vitro* dynamic systems have been used to test the effect of dietary components on microbial compositions and their metabolite production. Similar SCFA productions were found in the colonic compartments of the SHIME model when fermenting different fibre sources compared to the static *in vitro* faecal fermentation model [275]. However, no direct comparison of the *in vitro* model with *in vivo* measurements has been found.

The dynamic *in vitro* fermentation models described above only included microbiota in the colonic compartments. In addition, the models that include a small intestinal simulation only have one compartment for the small intestine, making it more challenging to introduce small intestinal microbiota as there are different numbers and dominant species in the different sections of the small intestine (Section 2.4.2). Recently, some studies have developed models that include ileal microbiota. The Smallest Intestine is a model that simulates the stomach and the various stages of the small intestine [276]. A mixture of seven bacterial strains (i.e., *Escherichia coli*, *Streptococcus salivarius*, *Streptococcus luteinensis*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Veillonella parvula*, and *Flavonifractor plautii*) is added to the ileal stage. These bacteria are chosen as they are thought to be prominent members of the human ileal microbiota. Another study has introduced ileal microbiota from human ileostomates into an *in vitro* model based on the TIM-1 and TIM-2 technology [277].

The SHIME model has been tested with ileal microbiota from pigs [278]. However, more optimization is required for the latter model as the ileal microbiota in the small intestinal compartment evolved to resemble the colonic compartment more closely than that of the ileal inoculum [278].

A limitation of the dynamic systems is that only a small number of samples can be tested simultaneously, with a maximum of eight replicates in the EnteroMix Colon Simulator [272] and ten in the TIM-2 [271]. Another limitation of these *in vitro* models is that they cannot mimic all *in vivo* aspects of the GIT, such as the mucosal layer, immune cells, and complex peristaltic movements. However, several studies have tried to incorporate a mucosal layer into the dynamic fermentation models to study the colonization and metabolism of the mucosal layer by GIT microbiota [279, 280]. It was found that the bacteria occupying the *in vitro* mucosal layer were similar to those found *in vivo* in the mucosa of healthy adult humans [281]. One model, the Host Microbiota Interaction (HMI) model, goes even further by allowing for the interaction between microbiota and host immune cells [282]. The HMI model consists of two compartments with gut microbiota and human enterocytes separated by a semi-permeable membrane coated with mucins. The membrane facilitates exchanges of metabolites between the microbiota and host cells. It allows small amounts of oxygen to enter the microbial compartment to simulate a microaerophilic environment in the mucosal layer.

In vitro fermentation models (both static and dynamic) offer excellent opportunities to better understand the GIT microbiota and its fermentation behaviour (including metabolite production) in a standardised and controlled environment with high reproducibility. These models allow studying the different steps of the fermentation process by repeated sampling and in multiple regions of the GIT. However, since the *in vitro* models cannot mimic all

physiological processes in the GIT and the host-microbe interaction, the results should be interpreted cautiously. Reliable comparisons between *in vitro* and *in vivo* data are needed to validate the *in vitro* results.

2.5.3. Combined in vivo/in vitro *models*

As described above, the *in vivo* model makes it difficult to determine total fermentation products due to absorption. I*n vitro* fermentation does not fully simulate GIT digestion. The latter is important in human nutrition as even ingredients considered ‘undigested’ might be affected by the gastric and small intestinal digestive processes, which changes their chemical properties such as solubility and viscosity [283], and the addition of endogenous material to the digesta. These, in turn, could affect the GIT fermentation of these undigested ingredients. Therefore, a methodology has been developed that combines the *in vivo* upper GIT digestion with *in vitro* fermentation. An *in vivo/in vitro* model for human hindgut fermentation has been developed and validated [284, 285]. The food of interest is fed to growing pigs, which then provide the ileal digesta that serves as a substrate for the *in vitro* hindgut fermentation. The ileal digesta is fermented with an inoculum prepared from human faecal material. Recently, a combined *in vivo/in vitro* ileal fermentation model has been developed and optimised using the growing pig as a model for the adult human [13]. In this methodology, the growing pigs were fed a human-type diet. They provided both the substrate (i.e., jejunal digesta) and the microbial inoculum (i.e., ileal digesta) to perform *in vitro* ileal fermentation. This methodology has demonstrated a significant amount of ileal fermentation and production of bacterial metabolites but has not yet been fully validated.

2.6. Concluding remarks

It is commonly known that the GIT microbiota plays an important role in breaking down food ingredients in the human GIT via microbial fermentation. The current understanding is that GIT fermentation occurs mainly in the large intestine, with little fermentation happening in the upper GIT [6, 7]. This belief is challenged by the reports of the ileal digestibility of dietary fibre (Table 2.4), the presence of a significant number of bacteria [43, 52] and the concentration of microbial metabolites [23, 185] in the human small intestine. It has been predicted that these small intestinal microbes could contribute to the fermentation of undigested food materials [12]. Most studies described above only reported one aspect of small intestinal fermentation, i.e., ileal digestibility of dietary fibre, small intestinal microbes, or metabolite concentrations in the small intestinal digesta. However, it is well established that the presence of specific bacterial species does not directly translate into how the ileal microbial community functions in fibre fermentation and fermentation outcomes. Therefore, it is important to study multiple aspects of small intestinal fermentation to better understand the actual contribution of small intestinal microbiota to the overall GIT fermentation. Due to the longer transit time and the higher number of bacteria in the ileum, it is suggested that most fermentation in the small intestine will be in the ileum. Therefore, the work reported here focuses on human ileal fermentation. Due to the limitations of *in vivo* studies, it is challenging to determine ileal fermentation, as certain aspects, such as metabolite production, cannot be directly measured *in vivo*. Therefore, a combined *in vivo*/*in vitro* ileal fermentation methodology has been developed and optimised [13].

The first aim was to validate the combined *in vivo*/*in vitro* ileal fermentation assay for the growing pig itself (**Chapter 3**) and for application to the adult human (**Chapter 4**). One of the main criticisms of using growing pigs as a model for adult humans is that the microbiota of pigs and humans are different, which is attributed to differences in environmental

conditions and diet. Therefore, it was expected that raising pigs in a more hygienic, non-farm environment and giving them human foods, and inoculating young piglets with an infant faecal extract, would improve the pig model (**Chapter 3**). It was expected that combining *in vivo* digestion with *in vitro* ileal fermentation using the growing pig as a model for the adult human is a valid and robust methodology for studying *in vivo* ileal fermentation. In addition, this thesis aimed to look at multiple aspects of ileal fermentation (i.e., the disappearance of fibre, microbial composition, and organic acid production) and compare these to hindgut fermentation (**Chapters 5 and 6**). This allowed the study of the relationship between specific bacteria in the ileal digesta and organic acid production when fermenting different fibre sources (**Chapter 7**). It was hypothesised that the small intestinal microbiota significantly contributes to the overall GIT fermentation in terms of the breakdown of undigested food and the production of microbial metabolites. Another understudied area of small intestinal fermentation is the effect of diet (i.e., one of the significant contributors to hindgut fermentation) on the ileal microbiota and their fermentation outcomes. The results presented in **Chapters 6 and 7** provide examples of how diet affects ileal microbiota and their fermentation. Like hindgut fermentation, it is expected that diet is an important contributor to the extent of small intestinal fermentation and its outcomes. The study reported in **Chapter 7** also quantified the contribution of both the inoculum and substrate to *in vitro* ileal fermentation.

Chapter 3: Validation of a combined *in vivo/in vitro* ileal fermentation assay in the growing pig

Author's contribution: AME Hoogeveen was responsible for the experimental design, ethical approval, the *in vivo* study (with technical assistance), the *in vitro* study, and sample analysis (with technical assistance). She also performed the data analysis, bioinformatics, statistical analysis (under the guidance of Carlos Montoya) and wrote the draft manuscript.

Abstract

Background. An *in vivo*/*in vitro* ileal fermentation assay using the growing pig has been developed but has not been formally validated.

Objective. This study aimed to validate the *in vivo/in vitro* ileal fermentation assay by comparing *in vitro* fermentation values with those obtained *in vivo* in the growing pig. The effect of raising pigs under different environmental conditions was also investigated.

Methods. Thirty piglets (1.59 ± 0.31 kg bodyweight, mean ± SD) were subjected to one of three treatments: artificially reared under hygienic conditions and receiving foods consumed by humans (AR), artificially reared plus human infant faecal extract inoculations (AR+), or conventionally reared on a farm (control). The AR+ piglets were orally inoculated from the first hours postnatally to postnatal day (PND) 8. From PND 7, the AR and AR+ pigs were raised in a hygienic non-farm environment, received infant formula for three weeks, and a human-type diet for five weeks. All pigs received a human-type diet with high fibre content at PND 63. On PND 78, pigs were euthanised, after which ileal digesta were collected to perform an *in vitro* ileal fermentation (*in vitro* organic matter (OM) fermentability and organic

acid production) and to determine digesta microbial composition and dietary OM fermentability *in vivo*.

Results. The rearing regimen resulted only in a few differences in ileal microbial composition. No differences ($P > 0.05$) were found in the α-diversity between the different rearing regimens. In general, the rearing regimen did not affect the *in vitro* production of individual organic acids. The *in vivo* and *in vitro* OM fermentability of proximal ileal digesta (19.7 ± 2.04% on average) was similar ($P > 0.05$) for the AR and control pigs but not for the AR+ pigs.

Conclusion. The control pigs provided the preferred and valid model for the *in vivo/in vitro* ileal fermentation assay.

3.1. Introduction

Recent studies have established the importance of small intestinal microbiota in gut fermentation in humans [12, 21] and monogastric animals [286, 287]. Small intestinal fermentation contributes to the production of metabolites that might affect the host's health [21, 286, 288]. A significant number of microbes are found in the human and pig small intestine, especially in the terminal small intestine (i.e., the ileum; e.g., 10^7-10^8 bacteria/g ileal effluent of human ileostomates [20, 286] and 10^8-10^9 bacteria/g ileal digesta of growing pigs [289, 290]). The ileal microbiota has many genes encoding for enzymes that metabolize carbohydrates, allowing the ileal microbiota to adapt rapidly to changing substrate availability [12]. Therefore, the small intestinal microbiota should be considered in gut fermentation. Only a few studies have investigated the human small intestinal microbiota and its role in overall gut fermentation because of limited accessibility to the human small intestine. It has been proposed that the growing pig could be used as an animal model for

ileal fermentation in the adult human, given the anatomical and digestive similarities observed in the upper gut (i.e., mouth to terminal ileum) [243]. Recently, Montoya *et al.*, 2018 [13] developed an ileal fermentation assay in growing pigs. This method combines *in vivo* digestion with *in vitro* fermentation. The pig is fed the food of interest and provides both the substrate (i.e., proximal ileal digesta comprising dietary and non-dietary materials) and the ileal inoculum (sourced from terminal ileal digesta) for the *in vitro* fermentation. The assay allows the determination of different fermentation parameters, such as organic matter (OM) disappearance and the production and absorption of organic acids, which are difficult to determine directly *in vivo*. Application of this assay has demonstrated that ileal microbiota differs from hindgut microbiota, both in composition and *in vitro* fermentative capacity [267, 286] and that they can be influenced by diets [287](Hoogeveen et al., submitted). The combined *in vivo/in vitro* approach is based on a hindgut fermentation model that has been validated and demonstrated to produce accurate results [284, 285, 291, 292], but to date, the combined *in vivo*/*in vitro* ileal fermentation model has not been fully validated. Therefore, the primary aim of the study was to validate the assay in the pig by comparing the *in vivo*/*in vitro* predictions of fermentation with *in vivo* values. It was hypothesised that the combined *in vivo/in vitro* model is a valid model for predicting ileal fermentation-based OM disappearance in the growing pig.

The study also investigated the effect on fermentation as determined using the assay, consequent upon raising the pigs under different environmental conditions. It is known, for example, that the composition of the gut microbiota differs between growing pigs and adult humans [44, 45, 247]. These differences may be at least in part due to differences in diet [122, 251] and environmental hygiene [252]. Therefore, it is important to determine if the *in vivo*/*in vitro* fermentation is affected by 'humanizing' the pig model. Does raising pigs in a clean non-farm environment and feeding the animals foods commonly consumed by

humans affect the modelled fermentation? Does inoculating the pigs at birth with human faecal extracts alter the predicted (*in vivo/in vitro*) fermentation? Inoculation with human microbiota has been suggested to improve the pig as a model for humans when looking at dietary modulation on microbiota and, therefore, gut fermentation [293]. Inoculation using faecal microbiota has been successfully applied to pigs and rodents [255, 294]. It has been demonstrated to shift the faecal microbiota composition to one more closely resembling that of the human donor. In this respect, the study aimed to compare the ileal microbial composition of conventional control pigs (i.e., pigs raised on the farm and fed a grain-based diet) with the ileal microbiota of pigs that were artificially reared (i.e., pigs raised in a more hygienic, non-farm environment and receiving a human-type diet), and the artificially reared pigs receiving oral inoculations of infant faecal extract in the first eight days of their life. The study also sought to evaluate whether such rearing regimens affected the *in vivo*/*in vitro* estimates of ileal OM fermentation and the pattern of organic acid production. It was hypothesised that both raising the pigs in a more hygienic, non-farm environment with a human-type diet and inoculating young pigs with infant faecal microbes would cause differences in ileal microbiota composition and OM fermentability as predicted using the *in vivo*/*in vitro* assay.

3.2. Materials and methods

The Massey University Animal Ethics Committee, Palmerston North, New Zealand, provided ethical approval for the animal study (protocol 19/116). The *in vivo* experiment was run in four phases (Figure 3.1), after which ileal digesta samples were collected to perform *in vitro* fermentations, determine the microbial composition, and *in vivo* OM fermentability.

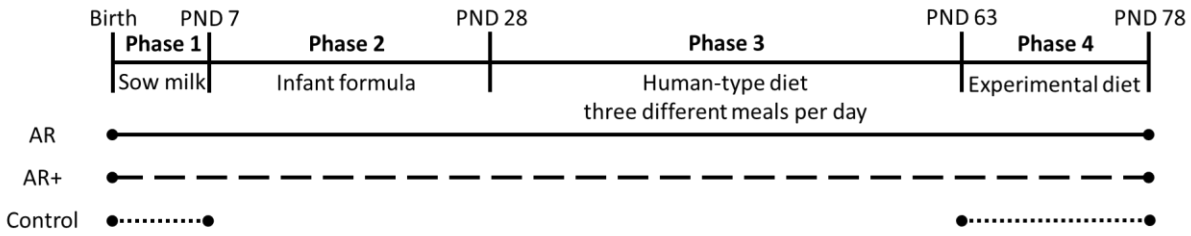

Figure 3.1: Study overview outlining the different phases of the study with the corresponding age of the pigs and the different diets provided to artificially reared (AR), artificially reared plus inoculated with infant faecal extract (AR+), and control (Phase 1 and 4 only) pigs. PND, postnatal day.

3.2.1. In vivo *experiment*

Phase 1. Thirty new-born male piglets (PIC Camborough 42 x PIC Line 337 boar; 1.59 ± 0.31 kg bodyweight (BW), mean ± SD) from five different litters (i.e., six piglets per litter) were selected based on birth weight (i.e., heaviest five male piglets per litter were selected) and were randomly divided into three experimental groups identifiable by coloured ear-tags: artificially reared (AR), artificially reared plus inoculation (AR+) or conventionally reared (control). The piglets stayed on the commercial farm with the sow to receive colostrum, allowing the natural development of their immune system and the gastrointestinal tract. The AR+ piglets received an infant faecal extract inoculation (1 mL/piglet) by gastric intubation whilst still with the sow, as described by Pang *et al.*, 2007 [255] (explained in detail in Supplementary methods). The first dosage was administered within the first 12 h after birth, then daily until postnatal day (PND) four, and then every other day until PND 8.

Phase 2. On PND 7, AR and AR+ piglets were transported to the Massey University Animal Physiology Unit, Palmerston North, New Zealand. The piglets were housed in metabolic crates (1.5 x 1.5 m) in a temperature-controlled room (28 ± 2°C) with a 16:8-hour light:dark

cycle at arrival. Each crate housed four piglets on the same treatment. From PND 22, the temperature was gradually reduced and reached 21°C on PND 28 (i.e., start Phase 3). The piglets were individually bowl-fed with a bovine-milk infant formula (Anmum NeoPro1, Fonterra NZ), prepared according to the manufacturer's instruction with added casein (0.6 g/100 mL formula) to match the daily protein intake provided by sow milk [295]. The infant formula was prepared fresh and warmed to 35°C in a water bath before feeding. The piglet's daily ration was 345 g liquid formula per kg BW per day [296, 297]. The feeding regimen and health assessment during Phase 2 are explained in detail in the Supplementary methods. From PND 22, a human-type diet from Phase 3 was gradually introduced to the piglets by mixing it with the infant formula in the bowl. The control piglets remained on the farm until Phase 4.

Phase 3. From PND 28, the pigs received a human-type diet, provided as three different meals per day at 0800, 1230, and 1700 h (i.e., breakfast, lunch, and dinner A; Supplementary Table 3.1). These meals were formulated to meet the nutrient requirements of starter pigs (i.e., 7-11 kg BW) as prescribed by the National Research Council, 2012 [298]. The daily ration was calculated as 90 g of DM per kg of metabolic BW ($BW^{0.75}$) divided by 20:40:40% DM over the three meals. From PND 45, the pigs received dinner B (replacing dinner A; Supplementary Table 3.1) to account for the change in nutrient requirement prescribed for the increased BW of the pigs (i.e., 11-25 kg BW) [298]. The pigs had free access to water for the duration of Phase 3. Pigs were weighed weekly, and the daily ration was adjusted accordingly.

Phase 4 (experimental phase). On PND 63, the control pigs were transported from the farm to the Massey University Animal Physiology Unit, as in previous studies [286, 287]. All pigs (i.e., AR, AR+, and control) were individually housed in metabolic pens (0.75 m^2) in a room maintained at 21 ± 2°C with a 10 h/14 h light/dark cycle. The pigs received the experimental

diet (Table 3.1). The experimental diet was formulated to have a higher fibre content (i.e., 7%) than the previous diet and to meet the nutrient requirements of the growing pig (25-50 kg BW) as prescribed by the National Research Council, 2012 [298]. Titanium dioxide (TiO_2) was included in the experimental diet as an indigestible marker. The pigs received the diet for 14 days, and their daily ration was calculated as 90 g of DM per kg of metabolic BW ($BW^{0.75}$) fed as three equal meals given at 0800, 1230, and 1700 h. The pigs had free access to water for the duration of Phase 4. Pigs were weighed weekly, and the daily ration was adjusted accordingly.

Sample collection. On PND 78, the pigs were given a single meal (i.e., one-third of their daily ration). Pigs were anaesthetised five hours post-feeding with a mixture of 0.04 mL/kg BW of Zoletil 100 (50 mg/mL; Provet NZ), Ketamine (100 mg/mL; Provet NZ), and Xylazine (100 mg/mL; Provet NZ) administered via intramuscular injection. Once anaesthetised, pigs were euthanised with an intracardiac injection of pentobarbital sodium (0.3 mL/kg BW; Pentobarb 500; Provet NZ). The time of euthanasia was chosen based on the highest observed flow of dry matter (DM) in the terminal ileum of ileal-cannulated pigs [299]. The small intestine was immediately dissected. The last 20% of the small intestine (i.e., the ileum) was cut into three pieces. The first 50 cm was defined as the proximal ileum, and the last 50 cm as the terminal ileum. The remaining length (177 ± 27 cm, mean ± SD) was defined as the mid-ileum. Digesta from the proximal ileum were collected to serve as a substrate for the *in vitro* fermentation assay and from the mid-ileum to prepare an inoculum for *in vitro* fermentation. A representative sample of the mid ileal digesta was collected for microbial analysis. Digesta from the proximal and terminal ileum (collected to determine *in vivo* OM loss) were collected for chemical analysis. Sample collection was done under a constant flow of carbon dioxide to maintain an anaerobic environment, and samples were stored at -80°C before further analysis.

Table 3.1: Ingredient and determined nutrient composition of the experimental diet given to all pigs in Phase 4 of the study[1]

	Amount
Ingredient, g/kg DM	
Milk (UHT, 3.3% fat)	130
Wheat biscuits (crushed)	183
Apple (raw, minced)	122
Egg (boiled, minced)	67
Baked beans (canned, minced)	96
Rice (white, cooked)	378
Premix of vitamins and minerals[2]	5.0
Titanium dioxide	3.0
Calcium carbonate (limestone)	10
Di-calcium phosphate	5.0
Nutrient, g/kg DM	
Ash	54.3
Crude protein	183
Total fat	109
Starch	379
Total dietary fibre	131
- Insoluble fibre	102
- Soluble fibre	29.0
Gross energy, MJ/kg	211

[1]The formulation of the diets was based on the chemical composition of the ingredients obtained from the New Zealand Food Composition Database (https://www.foodcomposition.co.nz/) and to meet the requirements of growing pigs respectively, as prescribed by the National Research Council, 2012 [298]; DM, dry matter; UHT, Ultra-high temperature.

[2]The vitamin and mineral premix (Pig Grower/Finisher Premix High copper, Nutritech, Auckland, New Zealand) supplied (per kg DM diet): vitamin A, 4.2 µg; vitamin D3, 0.075 µg; vitamin E, 47 mg; vitamin K3, 4 mg; thiamine, 3 mg; riboflavin, 6 mg; pyridoxin, 4 mg; vitamin B12, 30 µg; Biotin, 40 µg; Niacin, 30 mg; Pantothenic Acid, 22 mg; Folic Acid, 0.5 mg; Choline, 180 mg, Cobalt, 2 mg; Copper 250 mg; Iodine, 2 mg; Iron 160 mg; Manganese, 60 mg, Selenium, 0.6 mg; Zinc 230 mg.

3.2.2. In vitro *ileal fermentation assay*

The *in vitro* fermentation was performed according to an optimised assay developed by Montoya *et al.*, 2018 [13]. One inoculum was prepared for each pig by combining digesta from the mid-ileum with sterilised anaerobic Phosphate Buffered Saline (PBS; 0.1 M, pH 7.0, 4.1mM L-cysteine) at a ratio of 0.22:1 w/v. This mixture was homogenised with a hand-held blender (15 sec) before straining through four layers of sterile cheesecloth to remove larger particles. Fermentation bottles (i.e., 30 mL McCartney bottles) were prepared with either no substrate (i.e., blanks) or 100 mg substrate and 5 mL PBS. The substrates used for the *in vitro* fermentation were freeze-dried proximal ileal digesta of the same pig, arabinogalactan (AG; from larch wood; Sigma Aldrich, Auckland, New Zealand), fructooligosaccharides (FOS; from chicory root; Orafti P95, Beneo, Germany), and pectin (from citrus peel; Sigma Aldrich, Auckland, New Zealand). All preparations were done under a constant flow of CO_2 to maintain an anaerobic environment. After adding 5 mL inoculum, the fermentation bottles were flushed with CO_2, sealed, and incubated at 37°C for two hours [13]. For each substrate and blank, four fermentation bottles were used. Blanks were prepared for each inoculum to account for the potential fermentation of material in the inoculum (refer to calculations in the Supplementary methods).

3.2.3. Chemical analysis

The diets were analysed for DM (AOAC 930.15), ash (AOAC 942.05) [300], TiO_2 [301], crude protein (AOAC 968.06, nitrogen x 6.25), total fats using a Soxtec system (AOAC 2003.06), starch (α-amylase Megazyme kit, AOAC 996.11), total dietary fibre (including soluble and insoluble fibre; Megazyme, AOAC 991.43), and gross energy (using a Leco AC500 bomb calorimeter). The DM and ash content was measured in half the samples after *in vitro* fermentation (i.e., $n = 2$) to determine OM fermentability. The organic acids were quantified

in duplicate in the other half of the samples after *in vitro* fermentation (i.e., n = 2) using gas chromatography [302](Supplementary methods).

3.2.4. Microbial analysis

DNA was extracted from the digesta of the mid-ileum of individual pigs using the QIAamp PowerFaecal Pro DNA Kit (Qiagen, Australia). Before extraction, the samples were homogenised in a Mini-Beadbeater-96 (Biospec Products, Bartlesville, OK, USA) for 4 minutes before DNA extraction. A quantitative PCR method was used to quantify the total number of bacteria (i.e., 16S rRNA gene copies; Supplementary methods). The taxonomic composition was determined using 16S rRNA gene sequencing (i.e., Illumina sequencing; Supplementary methods).

3.2.5. Statistical analysis

For the *in vivo* study, a sample size of seven animals was calculated to be required to reach statistically significant ($P \leq 0.05$) differences in the taxa found in the faecal microbial community of germ-free piglets [256] and mice [303] receiving a human faecal inoculum compared with animals that did not receive an inoculum with a power >80% at a two-tail 5% significance level, based on means and variances reported in previous studies. However, because of the unknown effect of the infant faecal extract inoculation on the ileal microbial community and the duration of this study, a sample size of ten animals per treatment was used for this study.

The Bray Curtis Dissimilarity was calculated in MicrobiomeAnalyst [304, 305]. Its statistical significance was tested using the permutational multivariate analysis of variance

(PERMANOVA) and PERMDISPER tests. The SAS version 9.4 software (SAS Institute Inc.) was used for the remaining statistical analyses. Only microbial taxa with a relative abundance of 0.1% or higher in at least one sample were included in the statistical analyses.

The effect of the treatment (AR, AR+) and control regimens on the bacterial taxa and Shannon Diversity Index were tested with a one-way ANOVA. A two-way ANOVA was performed to test the effect of the rearing regimen, the method (*in vivo* and *in vitro*) or substrate (proximal ileal digesta, AG, FOS, and pectin), and the interaction between the rearing regimen and method (or substrate) on the OM fermentability and the *in vitro* production of organic acids. For all the analyses, the pig was the experimental unit, and litter was included in the models as a random effect.

The model diagnostics of each response variable were tested using the ODS Graphics and repeated statement of SAS. The bacterial taxa required a log10 transformation to achieve homogeneity of variance. A repeated statement in the Mixed Procedure of SAS was included in each analysis to allow testing for homogeneity of variance by fitting models with the Restricted Maximum Likelihood method and comparing them using the log-likelihood ratio test. The selected model for each response variable had similar studentised residuals (i.e., equal variances) across rearing regimens. The mean values were compared using the adjusted Tukey-Kramer test when the model's F-value was significant ($P \leq 0.05$). The mean values were considered statistically different if $P \leq 0.05$. The relationship between the *in vivo* and *in vitro* OM fermentability was evaluated with the Spearman correlation analysis.

3.3. Results

All pigs remained healthy, and a daily live weight gain of 134 ± 37 (mean ± SD), 329 ± 158 g/day, and 628 ± 138 g/day was observed during Phases 2, 3 (AR and AR+ pigs only), and 4 (all pigs), respectively.

3.3.1. In vivo *and* in vitro *organic matter fermentability*

The *in vivo* OM fermentabilities were not influenced ($P > 0.05$) by the rearing regimen (Figure 3.2). The *in vivo* OM fermentability determined from the proximal ileum to the terminal ileum was similar to the *in vitro* OM fermentability when proximal ileal digesta were fermented with an inoculum prepared from the mid ileal digesta for the AR and control pigs (19.7 ± 2.04% on average; $P > 0.05$). However, for the AR+ pigs, the *in vivo* OM fermentability was 3.2-fold greater ($P \leq 0.05$) than for the *in vitro* counterpart. A statistically significant ($P \leq 0.05$) correlation between *in vivo* and *in vitro* OM fermentability was found for the control pigs ($r = 0.738$, $P = 0.037$) but not for the AR or AR+ pigs.

The ability of the ileal microbiota to ferment (*in vitro*) different fibre substrates was also evaluated based on OM disappearance. In general, FOS was the most fermented substrate (on average 39.6 ± 1.50%; $P \leq 0.05$, Figure 3.3), while AG was the least fermented substrate (on average 23.0 ± 1.50%; $P \leq 0.05$). An effect of the rearing regimen was also observed. The *in vitro* ileal OM fermentability of the AR+ pigs was lower ($P \leq 0.05$) than that of the AR and control pigs.

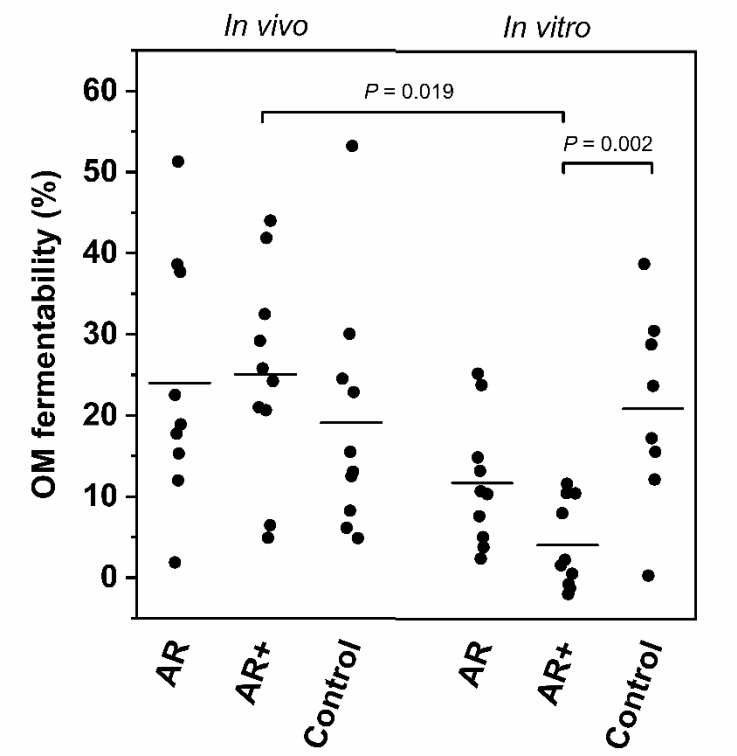

Figure 3.2: *In vivo* and *in vitro* OM fermentability (i.e., OM disappearance) of proximal ileal digesta of pigs reared under different environmental conditions and all fed a human-type diet for the last 14 experimental days. For the *in vitro* fermentation, each inoculum was prepared from the mid ileal digesta of the same animal. Data points represent individual samples, and the line represents the mean per treatment (*n* = 10 per treatment). A two-way ANOVA model was used to assess the effect of the rearing regimen, method (*in vivo* versus *in vitro*), and the interaction between the rearing regimen and method. AR, artificially reared; AR+, artificially reared inoculated with infant faecal extract; OM, organic matter.

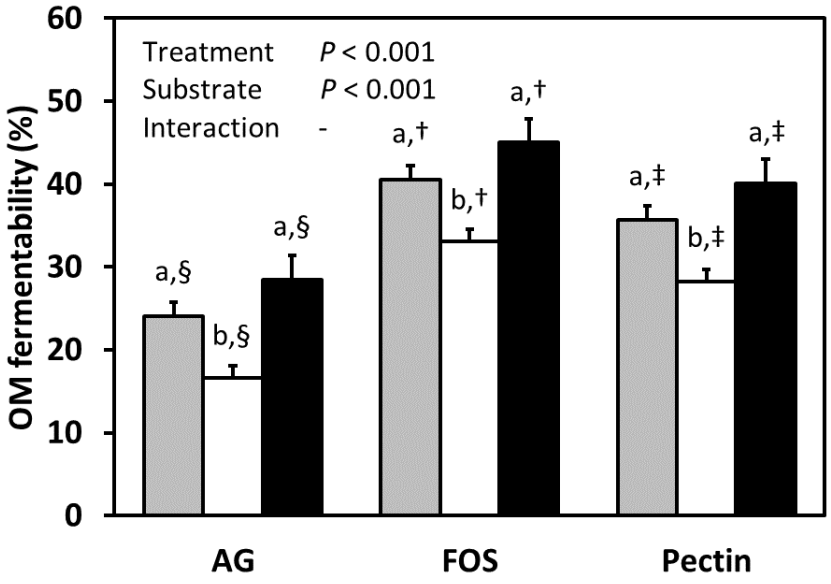

Figure 3.3: *In vitro* OM fermentability (i.e., OM disappearance) of fibre substrates inoculated with ileal microbiota obtained from pigs that were either artificially reared (grey), artificially reared plus inoculated with an infant faecal microbiota (white), or conventionally reared (control; black) and fed a human-type diet for the last 14 experimental days. Values are mean ± SEM, n = 10 animals per treatment. A two-way ANOVA model was used to assess the effect of the rearing regimen, substrate, and the interaction between the rearing regimen and substrate. The effect of the interaction between inoculum and substrate was not significant ($P > 0.05$) and therefore removed from the final model. Means with a different letter differ within the substrates ($P < 0.05$). Means with different symbols differ within the rearing regimens ($P < 0.05$). AG, arabinogalactan; FOS, fructooligosaccharides; OM, organic matter.

3.3.1. Ileal microbial composition

On average, the infant faecal extract had 8.36 ± 0.24 $\log_{10}$ 16S rRNA gene copies/g inoculum. The predominant phyla found in the human infant faecal extracts that were used

to inoculate the piglets were Firmicutes (39%), Actinobacteria (28%), Proteobacteria (19%), and Bacteroidota (13%) (Supplementary Figure 3.1) and the most abundant genera were *Bifidobacterium* (27%) and *Bacteroides* (13%), followed by *Escherichia_Shigella* (6.5%), *Veillonella* (6.5%), and an unclassified genus belonging to the Lachnospiraceae family (6.2%) (Supplementary Figure 3.2).

Five different bacterial phyla and 44 genera were identified (> 0.1% relative abundance in at least one sample) in the mid ileal digesta of the pigs at PND 78 (Supplementary Table 3.2). Of these genera, 63% (i.e., 28 genera) were present at a frequency of ≥80% in all treatments. Bacterial genera with a low frequency of occurrence (i.e., ≤30% in all treatments) were *Chlamydia*, *Megasphaera*, and an unclassified genus belonging to the family Pasteurellaceae. Some differences in the frequency of occurrence between the rearing regimens were observed (data not shown). For example, *Actinobacillus* had a greater frequency in the mid ileal digesta of AR+ pigs (60%) compared to the AR and control pigs (30 and 20%, respectively), and the AR and AR+ pigs had a greater frequency of *Cellulosilyticum* (60% for both treatments) than the control (30%). The genus *Mycoplasma* was present in all the control pigs but in none of the AR and AR+ pigs.

The rearing regimen did not affect the total number of bacteria (based on the number of 16S rRNA gene copies). However, the rearing regimen affected the number of gene copies belonging to seven bacterial genera (i.e., 16% of the total genera identified; Table 3.2). For example, a 4.4 and 3.0-fold greater ($P \leq 0.05$) number of *Actinomyces* was observed in the ileal digesta of AR+ pigs than in the ileal digesta of the AR and control pigs, respectively, based on back-transformed data. In the ileal digesta of the control pigs, the number of *Streptococcus* was 9.1- and 15-fold greater ($P \leq 0.05$) than in the ileal digesta of the AR and AR+ pigs, respectively. All the bacterial taxa identified in the ileal digesta are given in Supplementary Table 3.2.

The α-diversity (i.e., Shannon Diversity Index) was similar ($P > 0.05$) between the different rearing regimens (Figure 3.4). No significant separation for the groupings of the ileal samples was observed after performing the Bray-Curtis Similarity Principal Coordinates Analysis (i.e., β-diversity; Figure 3.5), supported by both PERMANOVA and PERMSDISPER tests ($P > 0.05$). In addition, the predicted metabolic functionality (i.e., KEGG pathways) of the microbial community in the ileal digesta of the pigs was not significantly ($P > 0.05$) different between rearing regimens (data not shown).

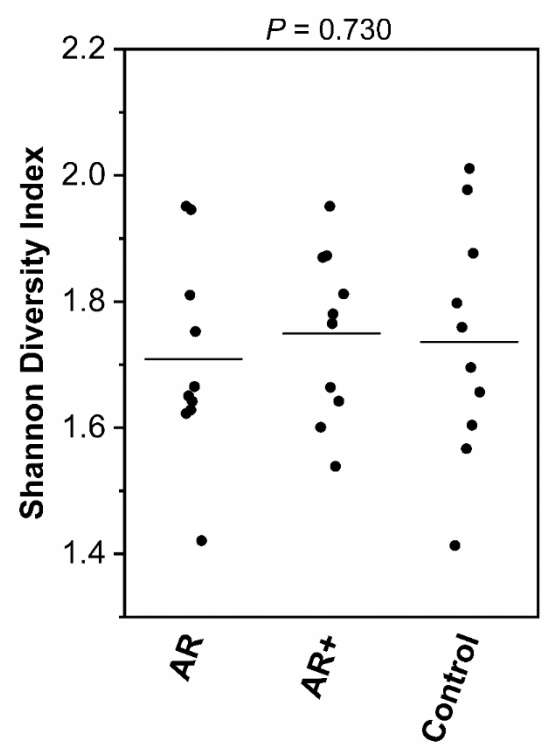

Figure 3.4: Shannon diversity index of ileal microbiota of pigs reared under different environmental conditions and all fed a human-type diet for the last 14 experimental days. Data points represent individual samples, and the line represents the mean per treatment (n = 10 per treatment). The effect of the rearing regimen was assessed using a one-way ANOVA test. AR, artificially reared; AR+, artificially reared plus inoculated with infant faecal extract.

Table 3.2: The number of bacteria of the taxa that showed a significant difference between the rearing regimens on in the ileal digesta of pigs reared under different environmental conditions and fed a human-type diet for the last 14 experimental days[1]

	Rearing regimen			
Phylum/Genus	**AR**	**AR+**	**Control**	***P* value**
	Log_{10} 16S rRNA gene copies/g DM digesta			
Actinobacteria	7.04 ± 0.192^{b}	7.51 ± 0.192^{a}	7.42 ± 0.192^{ab}	0.033
Actinomyces	6.43 ± 0.281^{b}	6.97 ± 0.224^{a}	6.48 ± 0.271^{b}	0.011
Bifidobacterium	4.20 ± 0.422^{b}	4.58 ± 0.351^{ab}	5.45 ± 0.390^{a}	0.020
Rothia	6.77 ± 0.207^{b}	7.23 ± 0.169^{a}	6.88 ± 0.221^{ab}	0.031
Firmicutes				
Lachnospiraceae_unclassified	5.52 ± 0.230^{ab}	5.87 ± 0.209^{a}	5.37 ± 0.198^{b}	0.045
Limosilactobacillus	7.77 ± 0.463^{ab}	8.43 ± 0.299^{a}	7.37 ± 0.352^{b}	0.029
Streptococcus	7.24 ± 0.244^{b}	7.36 ± 0.182^{b}	8.29 ± 0.224^{a}	0.001
Terrisporobacter	6.16 ± 0.491^{b}	6.75 ± 0.465^{ab}	7.98 ± 0.465^{a}	0.025

[1] Values are means ± SEM, n = 10 per treatment. Only taxa with >0.1% relative abundance in at least one sample and that had a significant (P < 0.05) rearing regimen effect (Supplementary Table 3.2) are presented. The number of 16S rRNA gene copies per taxa was obtained by multiplying the total number of 16S rRNA gene copies with the relative abundance of the taxa, with the assumption that each taxon has an equal number of 16S rRNA gene copies. Data were log_{10} transformed to achieve homogenous variance. A one-way ANOVA model was used to assess the effect of the rearing regimen. Means in a row with a different letter differ (P < 0.05). AR, artificially reared; AR+, artificially reared plus inoculated with infant faecal extract; ND, not detected.

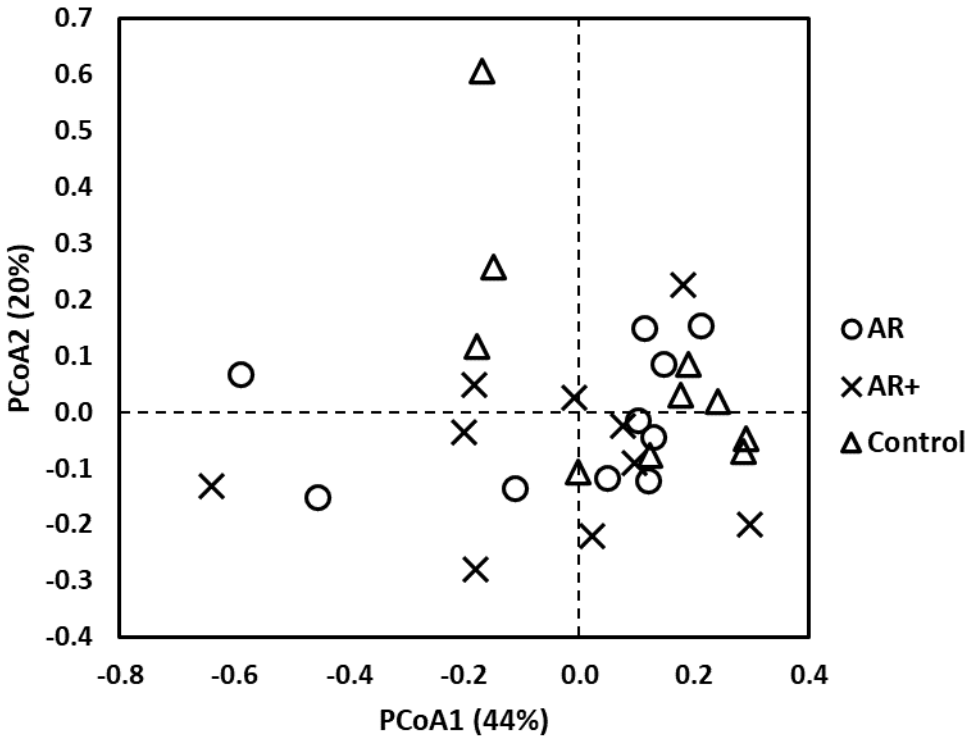

Figure 3.5: The Bray-Curtis dissimilarity of the ileal microbiota of pigs reared under different environmental conditions and all fed a human-type diet for the last 14 experimental days, displayed as a principal coordinate analysis (PCoA) plot. Individual symbols represent individual samples, *n* = 10 per treatment. The effect of the rearing regimen on the grouping was assessed using the permutational multivariate analysis of variance test (*P* = 0.12, F = 0.87, R^2 = 0.110). The homogeneity of the group dispersion was assessed using the PERMDISPER (*P* = 0.67, F = 0.68). AR, artificially reared; AR+, artificially reared plus inoculated with infant faecal extract.

3.3.1. In vitro *ileal organic acid production*

There was no significant interaction (*P* > 0.05) between the rearing regimen and substrate for the total organic acid production (i.e., the sum of formic, acetic, propionic, lactic, and succinic acid productions; Table 3.3). However, the total organic acid production was 1.1-

fold greater ($P \leq 0.05$) for the control pigs than for the AR+ pigs. The greatest ($P \leq 0.05$) total organic acid production after the *in vitro* ileal fermentation was for AG (on average 557 ± 13.8 mmol/kg DM substrate), and the lowest for FOS (on average 21.5 ± 2.29 mmol/kg DM substrate).

In general, the rearing regimen had no statistically significant ($P > 0.05$) effect on the production of individual organic acids. The only exception was that the control pigs had a 2.6- and 20-fold greater ($P \leq 0.05$) propionic acid production when fermenting FOS than the AR and AR+ pigs, respectively. The substrate affected the production of individual organic acids. For example, AG produced the greatest ($P \leq 0.05$) amount of formic, acetic, propionic, and succinic acids. Fermentation of the proximal ileal digesta led to the greatest lactic acid production ($P \leq 0.05$). The butyric, valeric, iso-butyric, and iso-valeric acid productions were negligible in the samples (i.e., below the detection limit) and, therefore, not reported.

3.4. Discussion

3.4.1. In vivo *and* in vitro *organic matter fermentability*

The primary aim of the study was to validate an *in vivo*/*in vitro* ileal fermentation assay. The *in vivo* OM fermentability of proximal ileal digesta was similar regardless of how the pig was reared, and the overall mean OM fermentability of the digesta for a human-type diet was 22.7 ± 2.66%. The agreement for *in vivo* OM fermentability across the three different rearing regimens demonstrates that, contrary to what was hypothesised, the way the pigs were raised had little effect on the fermentative capacity of the ileal microbiota. This finding is supported by observing only minor differences in ileal digesta microbial composition across the three rearing regimens (see below).

Table 3.3: Production of total and individual organic acids during *in vitro* fermentation of different substrates using inocula prepared from mid ileal digesta of pigs reared under different environmental conditions and fed a human-type diet for the last 14 experimental days[1]

Organic acid	Substrate	Rearing regimen			*P* value		
		AR	AR+	Control	Rearing regimen	Substrate	Intereaction[2]
		mmol/kg DM substrate					
Total	Digesta	232 ± 19.5^{ab,‡}	225 ± 19.5^{b,‡}	238 ± 19.5^{a,‡}	0.001	< 0.001	-
	AG	555 ± 14.5^{ab,†}	548 ± 14.5^{b,†}	561 ± 14.5^{a,†}			
	FOS	24.1 ± 3.66^{ab,ǁ}	17.1 ± 3.69^{b,ǁ}	30.1 ± 3.58^{a,ǁ}			
	Pectin	43.7 ± 3.34^{ab,§}	36.7 ± 3.46^{b,§}	49.7 ± 3.44^{a,§}			
Formic acid	Digesta	60.9 ± 7.89‡	39.2 ± 4.56‡	85.5 ± 23.8‡	0.080	<0.001	0.017
	AG	434 ± 23.0†	410 ± 14.5†	428 ± 11.9†			
	FOS	15.7 ± 2.46§	7.22 ± 0.977§	6.65 ± 0.3950‡			
	Pectin	9.67 ± 0.761§	12.7 ± 1.95§	10.3 ± 1.21‡			
Acetic acid	Digesta	53.8 ± 6.45‡	52.7± 6.45‡	51.9 ± 6.45‡	0.342	<0.001	-
	AG	67.1 ± 2.01†	66.0 ± 2.03†	65.2 ± 2.01†			
	FOS	5.32 ± 1.12§	4.23 ± 1.14§	3.40 ± 1.06§			
	Pectin	10.2 ± 0.972§	9.15 ± 1.05§	8.32 ± 0.963§			
Propionic acid	Digesta	1.24 ± 0.249†‡	1.72 ± 0.183†	1.45 ± 0.136†	0.714	<0.001	0.021
	AG	1.17 ± 0.173†	0.966 ± 0.095‡	1.32 ± 0.083†			
	FOS	0.358 ± 0.060‡	0.304 ± 0.072§	0.066 ± 0.130‡			
	Pectin	0.535 ± 0.091†‡	0.357 ± 0.059§	0.260 ± 0.052‡			

Lactic acid	Digesta	103 ± 9.22†	121 ± 19.5†	106 ± 10.8†	0.286	<0.001	0.018
	AG	48.8 ± 7.31‡	86.1 ± 13.3†	44.2 ± 5.75‡			
	FOS	8.06 ± 1.49^{b,§}	1.02 ± 2.80^{b,§}	20.8 ± 2.95^{a,‡}			
	Pectin	23.0 ± 2.30‡	16.6 ± 1.27‡	27.8 ± 3.63‡			
Succinic acid	Digesta	1.94 ± 0.458‡	1.81 ± 0.443‡	1.79 ± 0.352‡	0.938	<0.001	-
	AG	12.5 ± 0.443†	12.4 ± 0.428†	12.4 ± 0.323†			
	FOS	1.40 ± 0.462‡	1.27 ± 0.440§	1.25 ± 0.339‡			
	Pectin	1.57 ± 0.460‡	1.44 ± 0.451‡§	1.42 ± 0.354‡			

[1] Values are means ± SEM, *n* = 10 per treatment. A two-way ANOVA model was used to assess the effect of the rearing regimen, substrate, and their interaction for all organic acids. When the effect of the interaction between inoculum and substrate was not significant ($P > 0.05$), it was removed from the final model. Individual standard errors were required in the repeated statement to have similar studentised residuals as described in the statistical analysis section. Means in a row (i.e., rearing regimen effect) with different letters differ ($P \leq 0.05$), and means in a column (i.e., substrate effect) with different symbols differ ($P \leq 0.05$). The butyric, valeric, iso-butyric, and iso-valeric acid productions were negligible in the samples (i.e., below the detection limit) and, therefore, not reported. AG, arabinogalactan; AR, artificially reared; AR+, artificially reared plus inoculated with infant faecal extract; DM, dry matter; FOS, fructooligosaccharides.

[2] When the effect of the interaction between the rearing regimen and substrate was not significant ($P > 0.05$), it was removed from the final model.

When values for the *in vitro* fermentation of the proximal ileal digesta related to the ingestion of a human-type diet (i.e., the predicted OM fermentation values) were compared with the *in vivo* values (i.e., actual fermentation), differences were not statistically significant ($P > 0.05$) for the control and the AR pigs, but there was a relatively large and statistically significant ($P \leq 0.05$) difference between the *in vivo* and *in vitro* OM fermentability for the AR+ pigs. The *in vitro* OM fermentability of proximal ileal digesta obtained with the control pigs was similar to that of jejunal digesta of growing pigs raised conventionally and fed semi-synthetic diets [287] or a human-type diet [13] (22% versus 22% and 30%, respectively). It was concluded that the *in vitro* OM fermentation assay is a valid measure for determining the *in vivo* ileal fermentation, at least for the AR and control models.

The AR+ pigs showed poor agreement between *in vivo* and *in vitro* ileal OM fermentability, suggesting that the AR+ treatment was an invalid model for the *in vivo*/*in vitro* ileal fermentation assay. The reasons for the observed difference in *in vivo* and *in vitro* OM fermentability for the AR+ pigs are unclear. The difference may be related to the survival of some specific microbes during *in vitro* ileal fermentation for the AR+ pigs or the lower stability of this inoculum under *in vitro* conditions. In addition, the effect of non-bacterial members that could be part of the ileal microbial community (e.g., fungi and viruses) is unknown. Also, the microbial analysis used in this study was based on the presence of 16S rRNA genes that do not discriminate between viable and non-viable bacteria.

When the ileal inocula prepared from growing pigs that had been reared differently were used to ferment three fibre sources (AG, FOS, and pectin), known to be well fermented by small intestinal microbiota of pigs [267] and adult humans [21], there were no statistically significant ($P > 0.05$) differences in OM fermentability between the AR and control pigs, but the OM fermentability for the AR+ pigs was always lower ($P \leq 0.05$) than that for the AR and control pigs. This observation accords with the *in vivo* and *in vitro* comparisons. Given that

the *in vitro* OM fermentabilities of proximal ileal digesta, AG, FOS, and pectin did not differ ($P > 0.05$) between the AR and control pigs and that the *in vitro* OM fermentation values did not differ significantly ($P > 0.05$) from the *in vivo* values, it is concluded that both AR and control pigs are suitable models for the ileal fermentation assay. However, the control rearing regimen is simpler, easier, and less costly (both financially and ethically). Therefore, it is proposed as the appropriate approach for the *in vivo*/*in vitro* ileal fermentation assay.

3.4.2. The Ileal microbial community

Using a pig model to study the human gut microbiota and fermentation has been criticised since there are differences in the gut microbiota of pigs and that of adult humans [44, 45, 247]. Diet and environmental hygiene are considered to at least partly play a role in these species differences [122, 252]. However, the results of the current study show that both the total number of bacteria and individual taxa in the ileal digesta of pigs were similar despite different rearing conditions (i.e., artificially reared versus conventional reared). Therefore, a secondary aim of this study was to determine whether refinement of the animal model used in the ileal fermentation assay (i.e., artificially rearing in a non-farm environment and inoculation with an infant faecal extract) would cause changes to the ileal microbiota. Previous studies using DNA fingerprinting techniques have shown that inoculation of germ-free piglets with a human faecal inoculum successfully changed their faecal microbiota to more closely resemble the microbiota of the inoculum [255]. In the current study, however, the infant faecal extract inoculation of the AR+ pigs affected only a few genera in the ileal digesta. For example, *Actinomyces* and *Rothia* (both Actinobacteria) were increased in the AR+ pigs and therefore suggested to be influenced by the infant faecal inoculum.

The differences in ileal microbiota of the AR and AR+ pigs compared to the control pigs were lower than expected, which could be because the piglets were born naturally (i.e., not germ-free) and exposed to similar environmental microbiota on the farm during the first week (i.e., Phase 1). Secondly, ileal microbiota differs from faecal microbiota [8]. Ileal microbiota is more transient and highly adaptive to short-term changes, such as diet [20]. Therefore, it is likely that the ileal microbiota of the AR, AR+, and control pigs adapted similarly to the dietary changes in Phase 4. Unfortunately, because of the inaccessibility of the ileum, it was not possible to monitor the changes in ileal microbiota during the study. Thirdly, the microbiota for the inoculum was provided by mainly breastfed infants provided, which does not resemble the adult faecal microbiota. For example, greater numbers of *Bifidobacterium* are expected in infant faecal microbiota than in adult faecal microbiota [306]. Human adult faecal microbiota was found to establish themselves better in germ-free piglets than infant microbiota [258]. A fourth consideration is the duration of the study. The ileal microbiota was characterised on PND 78, which allowed the ileal microbiota to evolve for a considerable time after the inoculation was finished. It has been shown that one single infant faecal inoculation resulted in a change in the faecal microbiota of germ-free piglets within 24 hours, but this initial change diminished over the weeks after that [307]. Therefore, the study's duration may have contributed to the limited differences found in the ileal microbiota of the different rearing regimens.

Mycoplasma was only found in the control pigs, which was expected as it is a common gut bacterium for swine on a farm [247]. In addition, *Terrisporobacter*, *Streptococcus*, and *Bifidobacteria,* dominant bacteria within the porcine ileal microbiota [308-310], were found in greater numbers in the ileal digesta of the control pigs compared to the AR and AR+ pigs. This could be explained by the higher total dietary fibre content (14%) of the feed provided on the farm. For instance, *Bifidobacteria* in the ileal digesta has been positively correlated

with dietary fibre intake [309, 311]. Overall, these differences observed between conventional and artificially reared pigs suggest that the environmental conditions and diet influenced the ileal microbial community. However, these differences were minor, as supported by the similar diversity measures (i.e., Shannon Index, Bray Curtis) observed between the rearing regimens.

3.4.3. In vitro organic acid production

In line with what appeared to be relatively small differences in the ileal microbial composition across rearing regimens, the *in vitro* ileal production of the individual organic acids did not differ. This suggests that the different microbial communities had a similar capacity to produce organic acids, which accords with similar predicted metabolic functions (i.e., KEGG pathways). The lack of differences in the production of individual organic acids between the rearing regimens agrees with previous observations that the substrate was a greater contributor to the organic acid production than the microbial community (i.e., inoculum) (Hoogeveen et al., submitted). The organic acid production for the different substrates (i.e., AG having the greater formic, acetic, and succinic acids production) was similar to previous observations when fermenting them *in vitro* with ileal microbiota from pigs fed diets containing human foods [267](Hoogeveen et al., submitted). When total organic acids production was analysed, a statistically significant ($P \leq 0.05$) effect of the rearing regimen was seen, with the AR+ regimen supporting lower total organic acids production than the AR and control regimens for digesta, AG, and pectin.

3.5. Conclusion

Based on the results of this study, ethical cost, and the effort needed to rear the piglets artificially, it is concluded that using conventional pigs at PND 63 is a preferred model for future ileal fermentation studies. The similarities between *in vivo* and *in vitro* ileal OM fermentation observed for the conventional pigs support the validity of the *in vivo/in vitro* ileal fermentation method. AR+ pigs led to different *in vitro* OM fermentabilities and differences in total organic acid production, and there was poor *in vivo* and *in vitro* OM fermentability agreement for this pig model. It is possible that what appeared to be minor differences in the microbial composition of the inoculum contributed to this marked effect. Further work is warranted to compare the *in vitro* ileal fermentation between growing pigs raised conventionally and adult humans.

Chapter 4: Validation of an *in vivo*/*in vitro* ileal fermentation assay using the growing pig as an animal model for the adult human

Author's contribution: AME Hoogeveen was responsible for the experimental design, ethical approval, the *in vivo* animal study (with technical assistance), the *in vivo* human trial (with technical assistance), the *in vitro* study, and sample analysis (with technical assistance). She also performed the data analysis, bioinformatics, statistical analysis (under the guidance of Carlos Montoya) and wrote the draft manuscript.

Abstract

Background. An *in vivo*/*in vitro* ileal fermentation assay using the growing pig has not been formally validated for its application to adult humans.

Objective. The aim was to validate the ileal fermentation assay by comparing *in vitro* fermentation outcomes when fermenting fibre substrates with ileal inoculum prepared from growing pigs or adult human ileostomates.

Methods. Ten pigs (19 ± 4.5 kg bodyweight, mean ± SD) received a high-fibre diet containing human foods for two weeks. Ileal digesta were collected to determine the microbial composition and to perform an *in vitro* fermentation (organic matter (OM) fermentability and organic acid production) of arabinogalactan (AG), fructooligosaccharides (FOS), and pectin. Five human ileostomates incorporated the same human foods that made up the pig diet into

their daily diet for a week, after which they received two meals similar to those provided to the pigs. Ileal effluents were collected for *in vitro* fermentation and microbial analysis.

Results. The *in vitro* OM fermentability of AG, FOS, and pectin was similar ($P > 0.05$) between the pig and human ileal inocula (34.0 ± 2.13% on average). In general, the production of the individual organic acids was similar between humans and pigs ($P \leq 0.05$). Two-thirds of the bacterial genera found in the ileal digesta were similar between pigs and human ileostomates, which accords with the similar Shannon Diversity Index and predicted metabolic activity. The remaining genera were either host species or found to be different in numbers. For example, the number of *Veillonella* was 268-fold greater in human ileostomates than in pigs.

Conclusion. The *in vitro* ileal fermentation outcomes were similar across species despite some ileal microbial compositional differences, suggesting that the growing pig is a valid model for studying ileal fermentation in adult humans.

4.1. Introduction

The human small intestine harbours a significant number of microbes [20, 52]. For example, the ileum (i.e., the last part of the small intestine) contains 10^7-10^8 bacteria/g of ileal effluent in human ileostomates [20]. The small intestinal microbiota makes a quantitatively significant contribution to fermentation (i.e., the breakdown and metabolism of substrates under anaerobic conditions) in the human gut [12, 21]. The metabolites formed during fermentation can be directly absorbed from the ileum and likely contribute to intestinal function and health [21, 286, 288]. The human ileal microbiota has a different taxonomic composition from the faecal microbiota, with *Streptococcus* and *Veillonella* being the most abundant genera in the human ileum [20, 172, 183]. A considerable proportion of the genome of the ileal microbiota

is dedicated to metabolising carbohydrates, allowing the microbes to quickly adapt to the changing environment, such as substrate availability [12].

Even though it is known that the small intestinal microbiota contributes to gut fermentation, limited accessibility to the small intestine in humans makes it challenging to research this phenomenon. Therefore, and for this purpose, the growing pig has been suggested as an animal model for the adult human since the upper gut (i.e., mouth to terminal ileum) of the growing pig shows anatomical and digestive similarities to that of the adult human [243, 312]. Recently, a methodology that combines *in vivo* digestion in the pig and *in vitro* fermentation has been developed and optimised to investigate ileal fermentation [13]. Different fermentation parameters can be measured with this methodology, such as the disappearance of organic matter (OM) and metabolite production. The newly developed methodology has provided valuable insight regarding the differences in ileal and hindgut fermentation based on microbial composition and *in vitro* fermentation outcomes [267, 286]. The *in vivo*/*in vitro* methodology has shown merit, and upon validation, it can contribute novel information to the field of human nutrition. Previous work has shown that the *in vivo*/*in vitro* ileal fermentation model is a valid model for predicting *in vivo* ileal fermentation in the growing pig (Chapter 3). However, whether this model based on the growing pigs is suitable for predicting ileal fermentation in adult humans is yet to be demonstrated. To date, no research has been reported comparing the ileal microbial communities and their capacity to ferment fibre between pigs and humans fed a similar diet. Based on independent studies, the ileal microbiota of growing pigs [310, 313, 314] and adult humans [12, 20], although showing some similarities, do differ in taxonomic composition. It is hypothesised that the fermentative capacity and fermentation outcomes of both ileal microbial communities are similar despite these taxonomic differences and that the *in vivo*/*in vitro* ileal fermentation assay using the growing pig is a valid model for application to humans.

This study aimed to validate the *in vivo*/*in vitro* ileal fermentation assay using the growing pig as an animal model for the adult human by comparing the *in vitro* fermentation of different purified dietary fibre sources with an inoculum prepared from ileal digesta from growing pigs or ileal effluent from adult human ileostomates receiving a similar diet.

4.2. Materials and methods

4.2.1. Animal study

The protocol for the animal study was approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand (protocol 19/116). The experimental design for the animal study was described previously (Chapter 3). Based on the main outcome of that study (e.g., selection of an adequate animal model), only the control pigs were used in this study. Briefly, ten entire male pigs (PIC Camborough 42 x PIC Line 337 boar; 9-week-old; 19 ± 4.5 kg bodyweight (BW), mean ± SD) were received from a commercial farm and housed individually in metabolic pens. A high-fibre diet (7% total dietary fibre) containing human foods was provided to the pigs for two weeks (Supplementary Table 3.1). The diet met the requirements of the growing pig (25-50 kg BW) as prescribed by the National Research Council, 2012 [298]. Titanium dioxide (TiO_2) was added as an indigestible marker, but it was not considered in this study. The pigs were gradually introduced to the experimental diet over three days by mixing the diet with a commercial grower mix. The pigs were given their daily ration (i.e., 90 g of DM per kg of metabolic BW ($BW^{0.75}$)) divided equally over three meals at 0800, 1230, and 1700 h. After one week, the pigs were weighed, and their daily ration was adjusted accordingly. The housing was maintained at 21 ± 2°C with a 10 h light-14 h dark cycle, and the pigs had free access to water.

Sample collection. On day fourteen, the pigs received one single meal (i.e., one-third of their daily ration). Five hours after feeding, the pigs were anaesthetised with a cocktail containing Zoletil 100 (50 mg/mL; Provet NZ), Ketamine (100 mg/mL; Provet NZ), and Xylazine (100 mg/mL; Provet NZ). This cocktail was administered at a 0.04 mL/kg BW dose via intramuscular injection. After sedation, the pigs received a lethal dose of pentobarbital sodium (0.3 mL/kg BW; Pentobarb 500; Provet NZ) via intracardiac injection. Five hours post-feeding was chosen as the time of euthanasia as this coincides with the highest flow of DM observed in the terminal ileum of ileal-cannulated pigs [299]. Immediately after euthanasia, the small intestine was carefully dissected and removed from the body. The last 20% of the small intestine (i.e., ileum) was used for the study. The proximal ileum was defined as the first 50 cm of the ileum, and the terminal ileum was defined as the last 50 cm before the ileocaecal valve. The remaining length of the ileum (169 ± 22 cm length, mean ± SD) was defined as the mid ileum. For the *in vitro* fermentation assay, mid ileal digesta were collected to prepare the inoculum. An aliquot of the mid ileal digesta was collected for microbial analysis. All samples were collected under near anaerobic conditions (i.e., under a carbon dioxide flow) and stored at -80 °C until further analyses.

4.2.2. Human study

Five human ileostomates (Table 4.1) were recruited through the local ostomy societies (Manawatu and Wellington, New Zealand). The ileostomies were well established and functioning normally. All subjects reported being in good general health, without signs of small intestinal inflammation or dysfunction, and not having taken any antibiotics in the four weeks before the study. All participants gave their written informed consent to participate in the study, approved by the Massey University Human Ethics Committee, Palmerston North,

New Zealand (protocol SOA 20/43) and registered on the Australian New Zealand Clinical Trials Registry (ACTRN12622000813785).

Table 4.1: Overview of the characteristics of the human ileostomates included in this study[1]

Subject ID	Gender	Age	BMI (kg/m^2)	Duration since ileostomy surgery
H1	Female	68	23.4	36 years
H2	Male	71	24.8	~30 years
H3	Male	36	24.6	1 year and 8 months
H4	Female	75	21.1	30 years
H5	Male	37	24.1	3 years

[1]BMI, Body Mass Index.

The study consisted of an adaptation week and a study day. The subjects participated in the study while in their own homes. During the adaptation week, the participants were asked to incorporate the ingredients of the test meal (Table 4.2) into their usual diet. The minimum requirement was to consume four ingredients per day and every ingredient at least three times during the week. The compliance was recorded using a food diary. The adaption week aimed to adapt the ileal microbiota to the ingredients and mimic the adaption period of the pigs.

At 1800 h of day seven of the adaptation week, the participants consumed the test meal (Table 4.2) and were only allowed to drink water for the remainder of the night. After an overnight fast of 14 hours, participants received the same test meal on the study day at 0800 h and were requested not to drink or eat anything else for the remainder of the study unless stated. The participants replaced their ileostomy bags two hours post-meal (1000 h). Ileal effluents were collected for four hours (from 1000 to 1400 h) by changing the ileostomy bag hourly. The removed ileostomy bags were stored anaerobically (using the GasPak EZ pouch

system, BD, New Jersey, USA) in a cool box containing ice packs. Participants consumed 200 mL of water two hours post-meal (1000 h) and 200 mL of energy drink (E2 sports drink, The Coca-Cola Company, New Zealand) four hours post-meal (1200 h). After the fourth collection (1400 h), the ileal effluents from each participant (i.e., the contents of four ileostomy bags) were pooled and stored at -80°C until further analysis. The ileal effluent was used to analyse the microbial composition and to prepare an inoculum for the *in vitro* fermentation assay.

Table 4.2: The ingredients of the high-fibre test meal (7% total dietary fibre) provided to the human ileostomates[1]

Ingredient	Amount (g)
Milk (UHT, 3.3% fat)	243
Wheat biscuits (Weet-bix, Sanitarium, NZ)	42
Apple (raw)	148
Egg (boiled)	88
Baked beans (canned)	84
Rice (white, cooked)	164

[1]The formulation of the diets was based on the chemical composition of the ingredients obtained from the New Zealand Food Composition Database (https://www.foodcomposition.co.nz/). UHT, Ultra-high temperature.

4.2.3. In vitro *ileal fermentation assay*

The ileal fermentation assay was undertaken according to an optimised assay developed by Montoya *et al.*, 2018 [13]. An inoculum was prepared for each pig and human by combining mid ileal digesta or ileal effluent in a 0.22:1 w/v ratio with 0.1 M phosphate buffered saline (PBS. pH 7.0, sterile and anaerobic) containing 4.1 mM L-cysteine. After homogenisation, this mixture was filtered through four layers of sterile cheesecloth. PBS (5

mL) was added to each fermentation bottle with either 100 mg of fibre source (i.e., the substrate) or no fibre source (i.e., blanks). The fibre sources used for the *in vitro* fermentation were arabinogalactan (AG; from larch wood; Sigma Aldrich, Auckland, New Zealand), fructooligosaccharides (FOS; from chicory root; Orafti P95, Beneo, Germany), or pectin (from citrus peel; Sigma Aldrich, Auckland, New Zealand). A near-anaerobic environment was maintained by performing the preparations of the fermentation assay under a constant CO_2 flow. Inoculum (5 mL) was added to the fermentation bottles. It was flushed with CO_2, sealed, and incubated at 37°C for two hours [13]. Four fermentation bottles were used for each fibre source and blank. Blanks were included for each inoculum to account for the potential fermentation of material in the inoculum (refer to calculations in the Supplemental methods).

4.2.4. Chemical analysis

The chemical analysis of the diet given to the pigs included DM (AOAC 930.15), ash (AOAC 942.05) [300], gross energy (using a bomb calorimeter), crude protein (AOAC 968.06, nitrogen x 6.25), starch (α-amylase Megazyme kit, AOAC 996.11), total dietary fibre (including soluble and insoluble fibre; Megazyme, AOAC 991.43), total fat using a Soxtec system (Soxtec, AOAC 2003.06), and TiO_2 [301]. Half the samples after *in vitro* fermentation (i.e., $n = 2$) were subjected to DM and ash analysis to calculate OM fermentability (i.e., OM = DM – ash).

The organic acid content in the other half of the samples after *in vitro* fermentation (i.e., n = 2) was determined in duplicate using a gas chromatic methodology [302]. A mixture of formate, acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, lactate, and succinate was prepared and diluted at different concentrations for a standard curve. An internal standard, 2-ethylbutyric acid (5 mM, Sigma Aldrich), was added to the samples. N-

tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (Sigma-Aldrich) was added to derivatise the organic acids. The derivatised organic acids were quantified on a gas chromatograph (GC-2010, Shimadzu) equipped with a DB-1MS UI column (30 m x 0.25 mm x 0.25 µm, Agilent) and flame ionisation detector. Helium was the carrier gas. The blank fermentations (i.e., the bottles with no added fibre source) were used to correct the concentrations of the organic acids in the samples after *in vitro* fermentation (refer to the calculations in the Supplemental methods). An aliquot of an external control sample was included each time organic analysis was done to test the performance of the analysis. The control sample was prepared by combining *in vitro* ileal fermentation media of a previous study.

4.2.5. Microbial analysis

A mini-Beadbeater-96 (Biospec Products, Bartlesville, OK, USA) was used to homogenise for 4 min the ileal digesta of growing pigs and ileal effluent of human ileostomates. After homogenisation, the DNA was extracted using the QIAamp PowerFaecal Pro DNA Kit (Qiagen, Australia). The extracted DNA was subjected to a quantitative PCR methodology to quantify the number of bacteria (i.e., 16S rRNA gene copies; Supplemental methods). Additionally, Illumina sequencing of the 16S rRNA gene was conducted to obtain the taxonomic composition (Supplemental methods).

4.2.6. Statistical analysis

Based on mean values and variances of *in vitro* fermentability of different fibre sources (SD 14.4 %) and production of organic acids reported in previous studies with inocula prepared from pig ileal digesta [267] and human ileal effluent [21], five animals or participants (i.e.,

inocula) were required to detect a statistical difference (5 %) between the specie, with a power of >80% power at a two-tailed significance level ($P \leq 0.05$). The study reported here was part of a larger study with ten pigs per treatment (Chapter 3).

The statistical analyses were done using the SAS software (version 9.4; SAS Institute) unless stated otherwise. The effect of species (pig and human), substrate (AG, FOS, and pectin), and their interaction on the *in vitro* OM fermentability and *in vitro* production of organic acids was assessed with a two-way ANOVA model using the Proc Mixed procedure. The animal/participant was included as a random effect. The ODS Graphics and repeated statement were used to test the model diagnostics (including the normal distribution and homogeneity of variance) of each response variable. For some of the response variables, a repeated statement for species was required to have similar Studentised residuals. When the model's F-value was significant ($P \leq 0.05$), the mean values were compared using the adjusted Tukey-Kramer test. The mean values with $P \leq 0.05$ were considered statistically different.

Only taxa with >0.1% relative abundance in at least one sample were included in the taxonomic composition. The effect of species on the taxonomic composition, Shannon Diversity Index, and predicted metabolic activity was tested with an independent Student's *t* test. The ODS graphics and univariate procedures of SAS were used to evaluate the normal distribution of the *t* test. A $\log_{10}$ transformation was required to achieve homogenous variance for the taxonomic composition data. When the variance was unequal, the *P* value was obtained from the Satterthwaite separate variance *t* test. The statistical significance of the Bray Curtis Dissimilarity was tested in MicrobiomeAnalyst [304, 305] using the permutational multivariate analysis of variance (PERMANOVA) and PERMDISPER functions.

4.3. Results

All pigs remained healthy and had an average daily weight gain of 628 ± 138 g/day (mean ± SD). One human ileostomate had to repeat the experiment due to an intestinal blockage. When repeating, the subject only received the 0800 h meal on the study day (not the 1800 h meal on day seven of the adaptation week).

4.3.1. In vitro *organic matter fermentability*

The *in vitro* OM fermentability of the fibre sources (34.0 ± 2.13% on average; Figure 4.1) was similar ($P > 0.05$) between the inocula of growing pigs and human ileostomates. There was no statistically significant effect of the species inoculum. Across both species, FOS and pectin were more fermented (41.2 ± 2.43% and 37.5 ± 2.43%, respectively, $P \leq 0.05$) than AG (23.2 ± 2.63%).

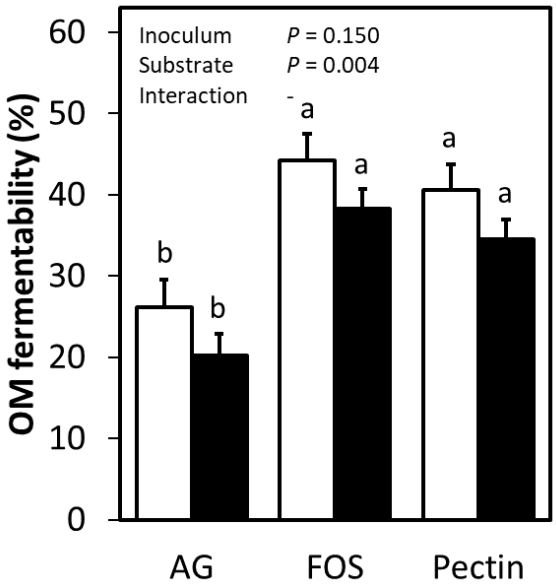

Figure 4.1: *In vitro* OM fermentability of different fibre substrates fermented with an inoculum prepared with ileal digesta obtained from growing pigs (white; $n = 10$) and ileal

effluent obtained from human ileostomates (black; $n = 5$) that received a similar diet. Values are mean ± SEM. A two-way ANOVA model was used to test the effect of inoculum, substrate, and the interaction between the inoculum and substrate. The effect of the interaction between inoculum and substrate was removed from the final model as it was not significant ($P > 0.05$). Means with a different letter differ across substrates ($P < 0.05$). AG, arabinogalactan; FOS, fructooligosaccharides; OM, organic matter.

4.3.2. In vitro *ileal organic acid production*

Only negligible amounts of butyric, valeric, iso-butyric, and iso-valeric acids were found in the samples (i.e., below the detection limit); therefore, these organic acids are not presented. On average across substrates, the pig ileal inoculum led to a greater ($P \leq 0.05$) total organic acid production (i.e., the sum of formic, acetic, propionic, lactic, and succinic acids) than the human ileal inoculum (Table 4.3). There was no difference between the species for total organic acid production for AG, but it was lower in humans for the pectin and markedly lower for FOS. No significant ($P > 0.05$) difference between the inocula from pigs and humans was observed for *in vitro* propionic and lactic acid production. However, species influenced the production of formic, acetic, and succinic acids, and this effect depended on the substrate fermented (Table 4.3). For example, the *in vitro* fermentation of FOS and pectin with the pig inoculum produced more ($P \leq 0.05$) formic acid than the human inoculum. On the other hand, the *in vitro* fermentation of FOS produced 2.4-fold greater ($P \leq 0.05$) acetic acid using the human inoculum compared to the pig inoculum. The substrate influenced the total organic acid production and the production of the individual organic acids. In general, *in vitro* fermentation of AG resulted in the greatest ($P \leq 0.05$) organic acid production, followed by FOS and pectin, regardless of the inoculum.

Table 4.3: Total and individual organic acid production during *in vitro* fermentation of different substrates using inocula prepared from mid ileal digesta of growing pigs and ileal effluent of human ileostomates receiving a similar diet[1]

		Species		*P* value[2]		
Organic acid	**Substrate**	**Pig**	**Human**	**Species**	**Substrate**	**Species x Substrate**
		mmol/kg DM substrate				
Total	AG	559 ± 14.9†	543 ± 15.1†	0.002	<0.001	-
	FOS	29.4 ± 2.94^{a,§}	13.3 ± 4.17^{b,§}			
	Pectin	48.4 ± 3.67^{a,‡}	32.3 ± 4.62^{b,‡}			
Formic acid	AG	428 ± 11.9†	395 ± 8.71†	0.010	<0.001	0.008
	FOS	6.65 ± 0.395^{a,‡}	3.57 ± 0.652^{b,‡}			
	Pectin	10.3 ± 1.21^{a,‡}	2.62 ± 0.379^{b,‡}			
Acetic acid	AG	69.3 ± 2.23†	67.6 ± 1.79†	0.509	<0.001	<0.001
	FOS	1.92 ± 0.269^{b,§}	4.59 ± 0.272^{a,‡}			
	Pectin	8.67 ± 0.996‡	5.65 ± 0.348‡			
Propionic acid	AG	1.29 ± 0.082†	1.35 ± 0.092†	0.387	<0.001	-
	FOS	0.101 ± 0.099‡	0.164 ± 0.108‡			
	Pectin	0.263 ± 0.045‡	0.326 ± 0.063‡			
Lactic acid	AG	44.2 ± 6.33†	67.7 ± 8.98†	0.903	<0.001	0.018
	FOS	20.3 ± 2.54‡	8.08 ± 3.59‡			
	Pectin	27.8 ± 2.54†‡	18.7 ± 6.01‡			
Succinic acid	AG	12.9 ± 0.589†	12.1 ± 0.616†	0.016	<0.001	-
	FOS	1.40 ± 0.254^{a,§}	0.590 ± 0.305^{b,§}			
	Pectin	1.45 ± 0.235^{a,‡}	0.635 ± 0.297^{b,‡}			

[1] Values are means ± SEM; *n* = 10 pigs and 5 humans. A two-way ANOVA model was used to assess the effect of treatment, substrate, and their interaction for all organic acids. The interaction between species and substrate was required as a repeated statement to have similar studentised residuals as described in the statistical analysis section for all the organic acids. Means in a row (i.e., species effect) with different letters differ ($P \leq 0.05$), and means in a column (i.e., substrate effect) with different symbols differ ($P \leq 0.05$). The butyric, valeric, iso-butyric, and iso-valeric acid productions were negligible in the samples (i.e., below the detection limit) and, therefore, not reported. AG, arabinogalactan; DM, dry matter; FOS, fructooligosaccharides.

[2] The effect of the interaction between species and substrate was not significant ($P > 0.05$) for the total and all individual organic acids. Therefore this effect was removed from the final model.

4.3.3. Ileal microbial composition

Six different bacterial phyla and seventy-five different bacterial genera were identified in the ileal digesta of growing pigs and the ileal effluent of human ileostomates at a relative abundance of 0.1% or more in at least one of the samples. About half of the identified genera were present in ≥80% of the samples obtained from both species. *Actinobacillus* and *Megasphaera* were two genera that occurred at a low frequency (≤ 40% of the samples of both species). A difference in the frequency of occurrence of genera was observed between the species (data not shown). For example, *Veillonella* was found in the ileal effluent of all the human ileostomates but in only 40% of the ileal digesta samples for the pigs. In addition, nine genera (e.g., *Scardovia* and *Solobacterium*) were exclusively found in human ileostomates, and three genera (e.g., *Mycoplasma*) were unique to the pigs.

The total number of bacteria (based on the number of 16S rRNA copies) in the ileal digesta of pigs and ileal effluent of humans was similar (Supplementary Table 3.2), with higher variability for the ileal effluent. Thirteen genera (i.e., 17% of the total genera identified) were found in different numbers between pigs and humans (Table 4.4). Ten of them were in greater numbers in the ileal digesta of growing pigs. For example, the number of *Lactobacillus* was 272-fold greater ($P \leq 0.05$) in the ileal digesta of growing pigs compared to the ileal effluent of the human ileostomates based on back-transformed data. *Veillonella* was one of the three genera with greater numbers in the ileal effluent of human ileostomates than in the ileal digesta of pigs (i.e., 268-fold greater, based on back-transformed data). All bacterial taxa identified in the study are presented in Supplementary Table **3.2**.

Table 4.4: Number of bacteria belonging to the taxa that differed between the ileal digesta of growing pigs and ileal effluent of human ileostomates that received a similar diet[1]

Phylum	Genus	Pig	Human	*P* value
		Log_{10} 16S rRNA gene copies/g DM digesta or effluent		
Firmicutes				
	Clostridiaceae_unclassified	6.68 ± 0.186	3.85 ± 0.797	0.022
	Clostridium_sensu_stricto_1	9.20 ± 0.177	5.19 ± 0.766	0.005
	Enterococcus	7.26 ± 0.191	5.31 ± 0.605	0.029
	Granulicatella	4.24 ± 0.276	6.16 ± 0.531	0.003
	Intestinibacter	5.34 ± 0.307	3.51 ± 0.901	0.030
	Lactobacillus	7.97 ± 0.296	5.54 ± 0.761	0.003
	Leuconostoc	8.78 ± 0.200	3.93 ± 0.883	0.004
	Romboutsia	8.39 ± 0.227	5.47 ± 0.880	0.027
	Terrisporobacter	7.96 ± 0.473	4.20 ± 0.794	0.001
	Turicibacter	8.23 ± 0.324	4.83 ± 0.741	<0.001
	Veillonella	4.47 ± 0.236	6.90 ± 0.843	0.042
Patescibacteria				
	Saccharimonadales_unclassified	5.34 ± 0.221	3.32 ± 0.658	0.003
	TM7x	4.02 ± 0.158	4.97 ± 0.476	0.033

[1] Values are means ± SEM; *n* = 10 pigs and 5 humans. The taxa shown in the table had a minimal relative abundance of 0.1% in at least one of the samples and were significant (*P* ≤ 0.05) different between pigs and humans. To calculate the number of 16S rRNA gene copies per taxa, the total number of 16S rRNA gene copies was multiplied by the relative abundance of the taxa, assuming an equal number of 16S rRNA gene copies per taxon. A log_{10} transformation of the data was needed to achieve homogenous variance. The effect of species was tested using an independent Student's *t* test. Means in a row with a different letter differ (*P* ≤ 0.05). DM, dry matter.

The Chao1 Diversity Index (i.e., richness or number of genera) was different (*P* ≤ 0.05). However, the Shannon Diversity Index, which considers the number of species (i.e., richness) and their relative abundance (i.e., evenness), was similar (*P* > 0.05) between the ileal microbiota from pigs and humans (Figure 4.2). The Bray-Curtis similarity principal coordinates analysis (i.e., β-diversity) showed a different grouping (*P* ≤ 0.05) of the ileal microbiota samples of pigs and humans, with similar group dispersions (*P* > 0.05) based on the PERMDISPER test (Figure 4.3). No significant difference (P > 0.05) was found in the

predicted metabolic functionality (based on KEGG pathways) of the ileal microbiota between pigs and humans (data not shown).

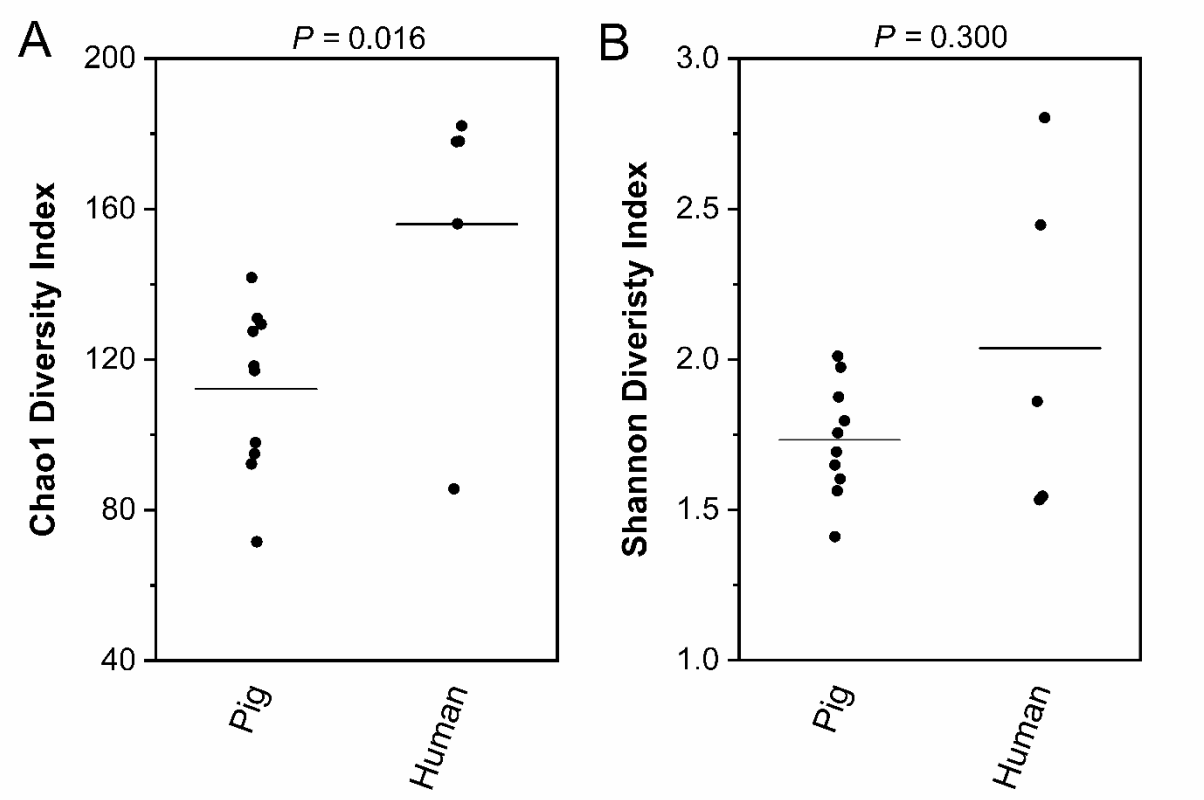

Figure 4.2: (A) Chao1 and (B) Shannon Diversity Indexes of ileal microbiota from growing pigs (*n* = 10) and ileal effluent obtained from human ileostomates (*n* = 5) that received a similar diet. Data points represent individual samples, and the line represents the mean per species. An independent Student's *t* test was used to test the effect of species.

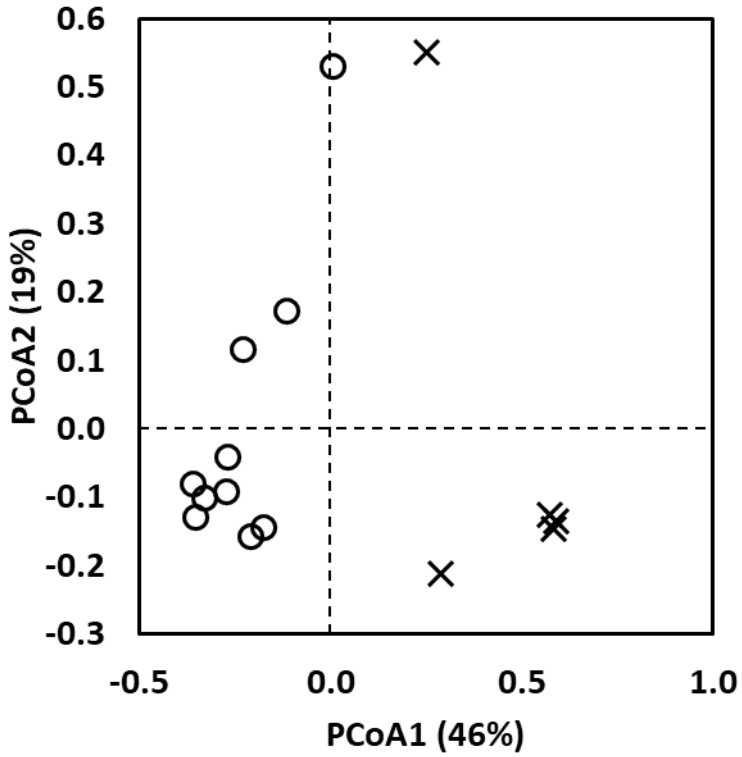

Figure 4.3: The β-diversity of the ileal microbiota of growing pigs (circles; *n* = 10) and human ileostomates (crosses; *n* = 5) that received a similar diet, based on the Bray-Curtis dissimilarity, and presented in a principal coordinate analysis (PCoA) plot. Individual symbols represent individual samples. A PERMANOVA was used to test the grouping of the different species ($P < 0.001$, $F = 10.04$, $R^2 = 0.436$), and the homogeneity of the group dispersion was assessed using the PERMDISPER ($P = 0.684$, $F = 0.173$).

4.4. Discussion

4.4.1. In vitro *organic matter fermentability*

The objective of this study was to determine whether an *in vivo*/*in vitro* ileal fermentation assay using the growing pig as an animal model for adult humans is valid for investigating ileal fermentation in adult humans. The use of pigs as an animal model to study the human gut microbiota and fermentation has been criticised by some due to reported differences in

the gut microbiota of pigs [310, 313, 314] and that of adult humans [12, 20]. As hypothesised, however, the present results for *in vitro* OM fermentability show that the ileal microbiota of pigs and humans ferment different fibre sources (AG, FOS, and pectin) to a similar degree. The similar degrees of fermentation also generally resulted in similar levels of organic acid production. These results, together with a documented similar digestive physiology in pigs and humans from the mouth to the end of the small intestine [243, 312], suggest that the *in vitro* ileal fermentation assay using the growing pig is valid for determining ileal fermentation in the adult human. The pig assay reliably predicts overall ileal OM fermentation but may now always accurately predict specific organic acid production.

Ileal microbiota from human ileostomates could ferment different fibre substrates (AG, FOS, and pectin), as shown elsewhere for similar substrates (FOS/inulin and pectin) but with longer fermentation times [21, 146]. Genomic analysis suggests that ileal microbial communities of adult humans are mainly driven by the metabolism of simple carbohydrates [12]. However, the current study demonstrates that ileal microbiota can also metabolise complex carbohydrates to a significant degree. Considering the present results (i.e., similar ileal fermentation outcomes between species and a significant degree of hydrolysis) and the species comparisons done between ileal and hindgut fermentation in the pig model (e.g., a similar degree of fermentation) [286], it is expected that ileal microbial communities make a significant contribution to the overall GIT fermentation in humans.

The current study included human ileostomates as they provide access to ileal microbial samples, and sampling is non-invasive. However, it has been argued that human ileostomates may not represent the ileal microbiota of intact adult humans due to potential exposure to oxygen [53] and changes in the anatomical structure after surgery. These claims are counteracted by high numbers of strictly anaerobic bacteria being found in ileal effluent [12, 315]. One study comparing the ileal effluent of human ileostomates with ileal samples

obtained via nasal intubation from healthy adult humans found similar bacteria between both ileal digesta samples [12]. Therefore, in the present study, it was assumed that ileal effluent obtained from human ileostomates accurately represents the ileal microbiota of adult humans in general.

4.4.2. In vitro *organic acid production*

The production of individual organic acids was similar between pigs and human ileostomates, with some exceptions (discussed below). This result suggests that the human and pig ileal microbial communities had a similar capacity to produce organic acids, which agrees with their similar predicted metabolic functions (i.e., KEGG pathways). The *in vitro* organic acid production for each fibre source followed a similar pattern as previously observed when ileal microbiota from pigs fed a diet containing human food was used to ferment these fibre sources [267](Hoogeveen et al., submitted). The current study found a sizeable formic acid production when AG was fermented *in vitro* with the pig and human ileal inocula (i.e., 42 to 150 times greater than when fermenting FOS or pectin), which accords with previous findings when AG was fermented *in vitro* using a continuous culture of *Bifidobacterium longum* [316] or pig ileal inoculum [267](Hoogeveen et al., submitted).

Minor differences between pigs and human ileostomates were found for individual organic acid productions. For example, greater acetic acid production was observed when FOS was fermented with the human ileal inoculum than with the pig inoculum. This result may be linked to the greater number of *Veillonella* present in the human ileal inoculum, as *Veillonella* is known to metabolise lactic acid to acetic acid [92].

4.4.3. Ileal microbial community

The main criticism of using pigs as an animal model to study gut microbiota and fermentation in humans is the different ileal microbial compositions between the species based on independent studies in growing pigs [310, 313, 314] and adult humans [12, 20]. However, in the present study, the total number of 16S rRNA gene copies and numbers of two-thirds of the identified genera were similar between the ileal digesta of pigs and ileal effluent of human ileostomates. This was supported by similar α-diversity numbers and predicted metabolic functionality. Nevertheless, it is important to highlight that the variability was higher in the ileal microbial community of humans, which could explain why no differences were observed across species for some microbial results. For example, the total number of bacteria was not statistically different across species despite there being 32-fold greater numbers (based on back-transformed data) in the ileal digesta of pigs compared to humans. The difference in variability between the species could be due to several factors, such as genetics, environment, and diet [122, 252], which are more standardised for pigs.

On the other hand, one-third of the genera were either unique to one species or in different numbers across species. For example, *Mycoplasma* was unique to the pig ileal digesta, which was expected as it is a common porcine gut bacterium [247]. *Streptococcus* and *Veillonella* were the most abundant genera in the ileal effluent of human ileostomates, which agrees with previous studies [12, 20, 183]. While *Streptococcus* was similar in the ileal digesta across species, *Veillonella* was present in greater numbers in the human ileal effluent. The significant difference in the Chao1 Diversity Index indicated that the human ileal microbiota had a higher number of species than the pig ileal microbiota. However, when considering the relative abundance of the genera (i.e., Shannon Diversity Index), the α-diversity of the ileal microbiota was similar for pigs and humans. In addition, it has been found that GIT microbiota from different individuals have a similar functional profile implying

functional redundancy, whereby different species can fulfil the same metabolic function within different microbial communities [317-320]. This may explain why the ileal microbiota of pigs and human ileostomates result in similar fermentation outcomes, even with differences in taxonomic composition.

Despite the difference in the taxonomic composition, the ileal microbiota from both species fermented the fibre sources to s similar degree. One study has reported similar *in vitro* fermentation outcomes (i.e., SCFA production) despite differences in faecal microbial populations [58]. However, in contrast, we have reported how different ileal microbial communities can influence *in vitro* fermentation outcomes (Hoogeveen et al., submitted). Based on correlations reported in our previous study, the difference in fermentation outcomes appears to be due to specific members of the ileal microbial community and the substrate used. For example, *Bifidobacterium* correlated positively with formic acid production when fermenting AG. In the present study, there was no difference in the number of *Bifidobacterium* and the production of formic acid across species. Considering the challenge of collecting ileal digesta in humans, the similar fermentation outcomes between ileal microbiota from pigs and human ileostomates found here indicate that the ileal digesta of growing pigs can be used to predict the ileal fermentation in humans. *In vitro* ileal fermentation is part of a combined *in vivo*/*in vitro* ileal fermentation methodology [13]. Using this combined *in vivo*/*in vitro* methodology, it has been demonstrated that ileal fermentation is as quantitatively important overall as hindgut fermentation [286] and that dietary interventions can modulate the amount of organic acids produced in the ileum [287](Hoogeveen et al., submitted).

4.5. Conclusion

Practically meaningful amounts of *in vitro* ileal fermentation were found in human ileostomates, highlighting the importance of an appropriate model for studying human ileal fermentation. The *in vitro* ileal fermentation outcomes using ileal digesta from the growing pig were similar to those obtained with ileal effluents of human ileostomates, suggesting that the growing pig is a valid model for ileal fermentation in adult humans.

Chapter 5: Ileal and hindgut fermentation in the growing pig fed a human-type diet

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Author's contribution: AME Hoogeveen was responsible for the data analysis, performed the statistical analysis (under the guidance of Carlos Montoya) and wrote the draft manuscript.

Abstract

Background. Dietary fibre fermentation in humans and monogastric animals is considered to occur in the hindgut but may also occur in the lower small intestine.

Objective. This study aimed to compare ileal and hindgut fermentation in the growing pig fed a human-type diet using a combined *in vivo/in vitro* methodology.

Methods. Five pigs (23 ± 1.6 kg (mean ± SD) body weight) were fed a human-type diet. On day fifteen, the pigs were euthanised. Digesta from terminal jejunum and terminal ileum were collected as substrates for fermentation. Ileal and caecal digesta were collected for preparing microbial inocula. Terminal jejunal digesta were fermented *in vitro* with an ileal digesta inoculum for 2 hours. In contrast, terminal ileal digesta were fermented *in vitro* with a pooled caecal digesta inoculum for 24 hours.

Results. The ileal organic matter fermentability (28%) was not different from hindgut fermentation (35%). However, the organic matter fermented was 66% greater for ileal fermentation than for hindgut fermentation (P = 0.04). The total numbers of bacteria in ileal and caecal digesta did not differ (P = 0.09). Differences ($P \leq 0.05$) were observed in the taxonomic composition. For instance, ileal digesta contained a 32-fold greater number of the genus *Enterococcus*. In contrast, caecal digesta had a 227-fold greater number of the genus *Ruminococcus*. Acetate and iso-valerate synthesis were greater ($P \leq 0.05$) for ileal fermentation than caecal fermentation, but propionate, butyrate, and valerate synthesis were lower. Short-chain fatty acids were absorbed in the GIT location where they were synthesised.

Conclusion. In conclusion, a quantitatively important degree of fermentation occurs in the ileum of the growing pig fed a human-type diet.

5.1. Introduction

Gastrointestinal tract (GIT) microbial fermentation is an important process in humans and monogastric animals. During fermentation, dietary fibre and non-dietary material are degraded by the GIT microbiota. Fermentation synthesises mainly short-chain fatty acids (SCFAs), organic acids such as lactate, and gasses such as CO_2, CH_4, and H_2 [1]. The SCFAs have beneficial health effects for the host, both locally within the GIT (for example, as an energy source for epithelial cells) [2] and systemically (e.g. regulation of glucose homeostasis) [3]. The current paradigm is that fermentation in humans and monogastric animals occur predominantly in the hindgut with little fermentation in the foregut [1].

There is a considerable number of microbes present in the foregut of the human and growing pig [8, 12, 321, 322]. These microbes may ferment dietary fibre, and several studies have reported important disappearance (i.e., fermentability) of dietary fibre at the end of the small

intestine in both human ileostomates and pigs [7, 145, 323, 324]. For example, 13% of the non-starch polysaccharides in potatoes [7] and 15 to 46% of the dietary pectin [145] disappeared in the foregut of human ileostomates. Several experiments also indicate low ileal digestibility values for dietary fibre in human ileostomates and ileal-cannulated pigs [141, 157, 324]. Montoya *et al.*, 2016 [325] discussed that non-dietary gut materials (such as mucin) might interfere with dietary fibre determination in ileal digesta and thus lead to underestimation of dietary fibre ileal digestibility. For instance, an estimate of the ileal digestibility of soluble fibre in kiwifruit increased by 50% when it was corrected for interfering non-dietary materials [144]. Consequently, dietary fibre fermentation in the foregut may be greater than is commonly believed.

The greater number and more diverse population of microbes in the ileum [8], coupled with a longer transit time of digesta in the lower small intestine, indicate that fermentation in the foregut may occur mainly within the ileum. Moreover, and based on functional genome analysis, the human ileal microbiota appears to be able to rapidly take up and metabolize simple carbohydrates (i.e., mono-, di- and oligosaccharides) [12]. This is important as transit time in the ileum is considerably shorter than in the hindgut [326]. In the growing pig, the adenylate energy charge in the last third of the small intestine was similar to that of the caecum, despite the lower number of anaerobic bacteria [327]. These observations indicate the potential for a quantitatively significant amount of fermentation occurring in the small intestine of humans and pigs. However, no reported studies have been able to quantify ileal fermentation due to the lack of a methodology to do so. It is hypothesised that ileal fermentation is as important as hindgut fermentation in terms of organic matter (OM) disappearance (i.e., fermentability) and synthesis of SCFAs despite difference in microbial population.

In this study, growing pigs were fed a human-type diet and ileal and hindgut OM fermentations were determined in the same animal using optimised and validated *in vivo*/*in vitro* fermentation assays that have shown to provide meaningful results to simulate fermentation in human adults [13, 284] (Chapter 3 and 4). This is the first study able to quantify OM fermentability, OM fermented, synthesis and absorption of SCFAs in the ileum. The quantitative importance of ileal fermentation was then assessed by comparing the ileal fermentation parameters with those obtained in the hindgut fermentation. The growing pig was used as an animal model for adult human foregut digestion [243, 328] because the collection of digesta in different locations of the small intestine is difficult and invasive in humans.

5.2. Materials and methods

5.2.1. In vivo *assay*

Dietary treatment. A high-fibre diet comprising foods commonly consumed by humans [329] was formulated to meet the nutrient requirements of the growing pig (National Research Council [330]) (Supplementary Table 5.1). Titanium dioxide (TiO_2) was added to the diet as an indigestible marker.

Animals housing and experimental design. Ethics approval for the animal trial was obtained from the Massey University Animal Ethics Committee (Palmerston North, New Zealand). The animal housing and experimental design were described previously in detail by Montoya *et al.*, 2018 [13]. Briefly, five nine-week-old entire male pigs (Hampshire x (Landrace x Large white), 23 ± 1.6 kg (mean ± SD) body weight (BW)) were housed individually in metabolism pens (1.5 x 0.5 m) in a room maintained at 24 ± 2.4°C with a 10 h/14 h light/dark cycle. Pigs received the experimental diet for 14 days, gradually adapting from a commercial diet to a

human-type diet during the first three days. The daily ration was 100 g dry matter (DM)/kg metabolic BW ($BW^{0.75}$) per day and given as two equal meals at 08.00 and 16.00 h. Pigs had free access to water during the study. Pigs were monitored during feeding. After feeding, cages were thoroughly washed, and toys were provided to the pigs. On day fifteen, pigs were fed half their daily ration as one meal and euthanised 5 hours postprandial by intracardial injection of sodium pentobarbitone (0.3 ml Pentobarb 300/kg BW; Provet). The small intestine was dissected out immediately and ligated into three equal parts. Digesta from the last 50 cm of the second (approximate terminal jejunum) and last (approximate terminal ileum) thirds of the small intestine were collected and used as substrates for the *in vitro* ileal and hindgut fermentation, respectively (Figure 5.1). Digesta from the remaining final third (i.e., last third minus terminal ileum) of the small intestine were collected along with caecal digesta for preparing microbial inocula for the *in vitro* ileal and hindgut fermentation, respectively. All digesta were collected in plastic bags containing carbon dioxide before being stored in insulated containers at 4°C to minimize bacterial activity while weighing the fresh digesta substrates. Representative samples of terminal jejunal digesta, terminal ileal digesta, and faeces were collected in Eppendorf tubes and stored at –20°C to determine the concentration of SCFAs. For the microbial analysis, aliquots from the ileal (i.e., last third minus last 50 cm) and caecal digesta were collected in Eppendorf tubes and stored at –80°C. Terminal jejunal and terminal ileal digesta and faeces were also collected, stored at –20°C, freeze-dried and finely ground for determining DM, OM, and TiO_2.

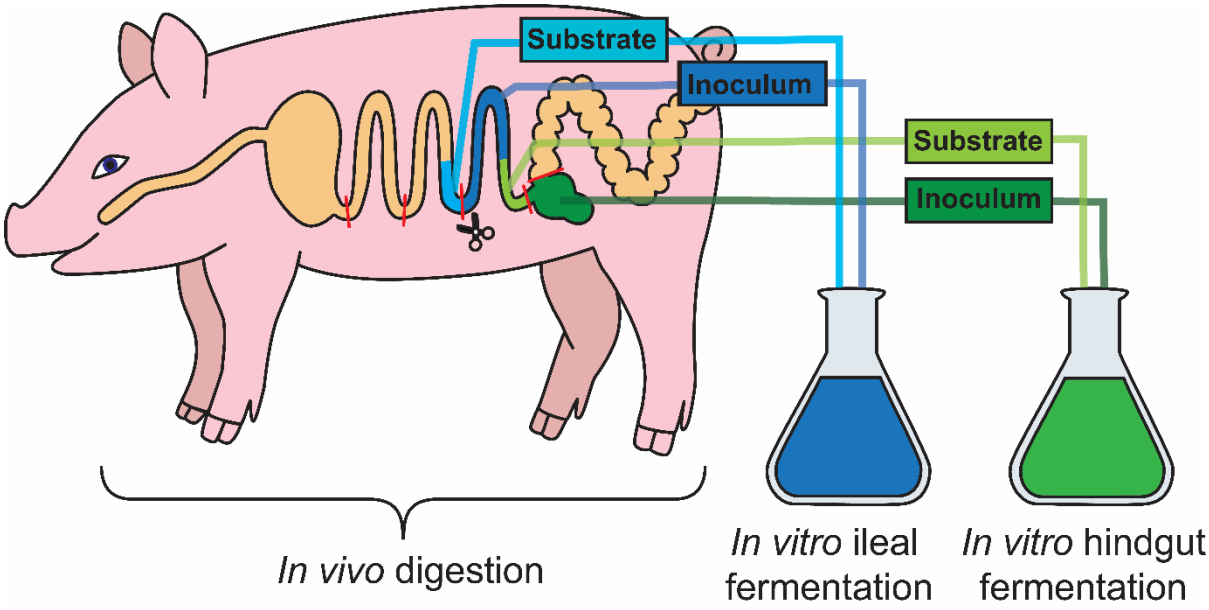

Figure 5.1: Schematic overview of the *in vivo*/*in vitro* ileal and hindgut fermentation methodology.

5.2.2. In vitro *fermentation assays*

A combined *in vivo*/*in vitro* methodology was used based on the growing pig. The pig provided both the substrate (terminal jejunal and terminal ileal digesta) entering each of the fermentation sections (ileum and hindgut) and the microbial inocula (ileal and caecal digesta) for the ileal and hindgut fermentation, respectively [13]. The substrate and inoculum of each fermentation section are then fermented *in vitro* to determine OM fermentability and synthesis of SCFAs. The *in vivo* and *in vitro* results were combined to predict amounts of OM fermented and the synthesis and absorption of SCFAs.

The combined *in vivo*/*in vitro* ileal and hindgut fermentation assays were optimised for different parameters such as incubation time, amount of digesta, pH, and medium elsewhere [13, 284]. For example, the incubation time (1 to 7 hours) did not significantly change the ileal OM fermentability using the *in vivo*/*in vitro* methodology [13].

Ileal fermentation. The *in vitro* ileal fermentation was performed according to Montoya *et al.*, 2018 [13]. The inoculum was prepared by pooling ileal digesta (digesta from the final third of the small intestine minus the last 50 cm) from all pigs and mixing with a sterilised anaerobic 0.1 M phosphate buffer saline (PBS) solution (4.1 mM L-cysteine, pH 7). The ratio digesta:PBS was 0.22:1, w:v. All bottles containing 5 ml of PBS either alone (blanks) or 1 g of the fresh substrate (i.e., terminal jejunal digesta for each of the five pigs) were inoculated with 5 ml of the ileal inoculum. A total of six bottles per pig were used. The ileal fermentation was conducted anaerobically at 37°C for 2 hours.

Hindgut fermentation. The *in vitro* hindgut fermentation was performed according to Coles *et al.*, 2013 [284]. The inoculum was prepared by pooling caecal digesta from all pigs and mixing them with a sterilised anaerobic 0.1 M PBS solution (4.1 mM L-cysteine, pH 7). The ratio digesta:PBS was 0.33:1, w:v. All bottles containing either 5 ml of PBS alone (blanks) or with 1 g of the fresh substrate (i.e., terminal ileal digesta for each of the five pigs) were inoculated with 5 ml of the ileal inoculum. A total of six bottles per pig were used. Hindgut fermentation was conducted anaerobically at 37°C for 24 hours.

After ileal and hindgut fermentation, the contents of three bottles were analysed to determine the concentration of SCFAs. The remaining three bottles were autoclaved (121°C for 20 minutes) to inactivate the bacteria and remove fermentation products before OM determination. The values of the three bottles for the concentration of SCFAs and OM determination were averaged per pig. Thus, the number of replicates was five for both ileal and hindgut fermentation.

5.2.3. Chemical analysis

The diet and substrate materials were analysed in duplicate for DM, ash, and OM (DM – Ash), TiO_2 [301], starch (Kit AA/AMG, Megazyme), crude protein (N x 6.25; using a LECO elemental analyser) [300], and lipids (by Soxhlet extraction using petroleum ether) [300]. The diet was also analysed for gross energy (using a LECO AC-350 Automatic Calorimeter) and soluble and insoluble dietary fibre [331]. DM, ash, and OM (DM – Ash) contents were also determined on the material remaining after the *in vitro* fermentation. The concentration of SCFAs was determined in the terminal jejunal digesta, terminal ileal digesta, faeces, and in the samples after *in vitro* fermentation, as described previously [292], with iso-caproic acid as an internal standard.

5.2.4. Microbial analysis

DNA extraction. DNA was extracted from ileal and caecal digesta (0.25 g) using the DNeasy Powersoil kit (QIAGEN), with alterations described by Healey *et al.*, 2017 [332]. Before extraction, the sample was homogenised in bead tubes (0.1 mm and 0.5 mm mix in bead solution) using a FastPrep-24 5G instrument (MP Biomedicals) at 5.5 m/s for three 60-second cycles with 5 minutes of rest on ice in between. Extracted DNA was quantified and quality-checked on a Qubit fluorometer (Invitrogen) and QIAxpert spectrophotometer (QIAGEN), respectively.

Quantitative PCR. Escherichia coli (Nissle) was used as a representative bacterium for the total bacteria and was grown in tryptic soy broth (Oxoid) at 37°C aerobically. Cell density was determined using a haemocytometer (Neubauer) and the culture was concentrated to $1.0x10^9$ cells/ml. DNA was then extracted as described above. A standard curve was constructed using 1:10 dilutions of the extracted standard DNA. Samples and standards

were run in triplicate by absolute quantification on the Light Cycler 480 real-time PCR instrument (Roche). SYBR Green I Master Mix (Roche) detection chemistry was used to detect double-stranded DNA amplification. The total reaction volume was 20 µl, consisting of 10 µl SyBr Green I Master mix, 4 µl forward primer (2.5 µM), 4 µl reverse primer (2.5 µM) and 2 µl DNA template or sterile water (blank). Each qPCR run included one activation cycle (95°C, 5 minutes), 32 to 40 run cycles [including denaturation (95°C, 30 seconds), annealing (60°C, 60 seconds) and extension (72°C, 60 seconds)], and one melt curve cycle (60 to 95°C at 0.1°C/s with continuous fluorescence acquisition) followed by a cooling cycle (40°C). The melt curve cycle enabled the differentiation between the target product and non-specific double-stranded products such as primer-dimers. The universal primers used were forward '(5'- TCCTACGGGAGGCAGCAGT) and reverse (5' – GGACTACCAGGGTATCTAATCCTGTT) [333].

16S rRNA gene sequencing and bioinformatics. Purified DNA from each sample was sent to the Massey Genome Service (Massey University, Palmerston North, New Zealand). The samples underwent library preparation as previously described[334] using primers that amplified the V3-V4 hypervariable region of the 16S rRNA gene, 16SF_V3 (5' - AATGATACGGCGACCACCGAGATCTACAC-index-TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). The library was pooled at equal concentrations and run on one lane of an Illumina MiSeq instrument using 2x250 bp paired-end chemistry. Quantitative Insights Into Microbial Ecology (QIIME) software V1.8.0 was used to analyse the Illumina MiSeq sequencing data [335]. PANDASeq was used with parameters of at least 40 bp overlap, a minimum of 350 bp length and a maximum of 500 bp length to assemble the forward and reverse reads into a continuous sequence. Chimeras

were filtered from the sequences and the reads clustered into operational taxonomic units based on a 97% identity threshold using USEARCH (-cluster_fast command with default parameters) [336, 337]. Sequence alignment was carried out using PyNAST with reference to the Greengenes database (version 13_8) [338]. The resultant OTU table was denoised by removing taxa with fewer than five total sequences across all samples. Alpha rarefaction was calculated using Faith's Phylogenetic Diversity metric [339] to a rarefaction depth of 10,000 sequences. Beta diversity was determined using Euclidean distances as input to generate principal coordinate plots. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the functional profiling of ileal and caecal microbial communities.

5.2.5. Calculations

Due to different amounts of DM entering the ileum and hindgut, data were normalised for dietary DM intake based on the ratio of the indigestible marker in the diet and digesta (i.e., data were expressed per kg diet DM intake). Normalizing parameters allowed comparing gut locations, as measures were expressed in the same unit. The calculations used for determining *in vitro* OM fermentability [292] and *in vivo*/*in vitro* fermented OM for either ileal or hindgut fermentation were as follows:

1) $\text{OM fermentability}_{\textit{in vitro}}\,(\%) = (\text{OM}_{\text{before } \textit{in vitro} \text{ fermentation}} - [\text{OM}_{\text{after } \textit{in vitro} \text{ fermentation}} - ((\text{OM}_{\text{blank initial}} + \text{OM}_{\text{blank final}})/2)]) / \text{OM}_{\text{before } \textit{in vitro} \text{ fermentation}} \times 100$
2) $\text{Fermented OM}_{\textit{in vivo/in vitro}}\,(\text{g/kg diet DM intake}) = \text{OM fermentability}_{\textit{in vitro}} / 100 \times (\text{TiO}_{2\text{-diet}}/\text{TiO}_{2\text{-terminal jejunal or terminal ileal digesta}})$

where $\text{OM}_{\text{blank initial}}$ and $\text{OM}_{\text{blank final}}$ are the amounts of OM in the blanks prior to (initial) and after (final) *in vitro* fermentation, respectively. *In vitro* ileal and hindgut fermentation had their

own blanks. $TiO_{2\text{-diet}}$ and $TiO_{2\text{-terminal jejunal or terminal ileal digesta}}$ are the TiO_2 concentrations (g/kg DM) in the diet and digesta, respectively. The $TiO_{2\text{-terminal jejunal digesta}}$ was used for the *in vitro* ileal fermentation, whereas $TiO_{2\text{-terminal ileal digesta}}$ was used for the *in vitro* hindgut fermentation.

The normalised total number of bacteria and archaea and number per phyla or genus, and the predicted metabolic activity in ileal and caecal digesta were calculated as follows:

3) Normalised total number of bacteria and $archaea_{\text{ileal or caecal digesta}}$ (16S rRNA gene copy number/kg diet DM intake) = number of bacteria and $archaea_{\text{ileal or caecal digesta}}$ (16S rRNA gene copy number/kg DM) x ($TiO_{2\text{-diet}}$/$TiO_{2\text{-terminal ileal or caecal digesta}}$)
4) Normalised number of bacteria or archaea per $phylum/genus_{\text{ileal or caecal digesta}}$ (16S rRNA gene copy number/kg diet DM intake) = normalised total number of bacteria and $archaea_{\text{ileal or caecal digesta}}$ (16S rRNA gene copy number/kg diet DM intake) x $\text{relative abundance}_{\text{phylum/genus}}$ (%) / 100
5) Normalised predicted metabolic $activity_{\text{ileal and caecal digesta}}$ (activity/kg diet DM intake) = relative activity/kg DM x ($TiO_{2\text{-diet}}$/$TiO_{2\text{-terminal ileal or caecal digesta}}$)

The synthesis of SCFAs during *in vitro* ileal and hindgut fermentation, estimated *in vivo*/*in vitro* synthesis of SCFAs (representing the estimated ileal or hindgut synthesis based on the amount of dry matter entering either the ileum or hindgut per kg DM diet intake), normalised concentration of SCFAs in terminal jejunal digesta, terminal ileal digesta and faeces, and estimated *in vivo*/*in vitro* disappearance of SCFAs in ileum and hindgut were determined as described previously [292], using the following equations:

6) Synthesis of SCFAsileal or hindgut *in vitro* (mmol/kg substrate DM incubated) = (SCFAsafter $_{\textit{in vitro}}$ fermentation (mmol/kg DM) – $SCFAs_{\text{jejunum or terminal ileum digesta}}$ (mmol/kg DM) – [($SCFAs_{\text{blank initial}}$ + $SCFAs_{\text{blank final}}$)/2])

7) Estimated synthesis of $SCFAs_{ileal\ or\ hindgut\ \textit{in\ vivo/in\ vitro}}$ (mmol/kg diet DM intake) = synthesis of $SCFAs_{ileal\ or\ hindgut\ \textit{in\ vitro}}$ (mmol/kg substrate DM incubated) x ($TiO_{2\text{-diet}}/TiO_{2\text{-terminal jejunum or terminal ileal digesta}}$)

8) Normalised concentration of $SCFAs_{terminal\ jejunum,\ terminal\ ileum\ digesta\ or\ faeces}$ (mmol/kg diet DM intake) = $SCFAs_{terminal\ jejunum,\ terminal\ ileum\ digesta\ or\ faeces}$ (mmol/kg DM) x ($TiO_{2\text{-diet}}/TiO_{2\text{-terminal jejunal or terminal ileal digesta or faeces}}$)

9) Estimated disappearance of SCFAs (mmol/kg diet DM intake) $_{ileal\ or\ hindgut\ \textit{in\ vivo}}$ = Normalised concentration of $SCFAs_{terminal\ jejunum\ or\ terminal\ ileum\ digesta}$ (mmol/kg diet DM intake) + estimated synthesis of $SCFAs_{ileal\ or\ hindgut\ \textit{in\ vivo/in\ vitro}}$ (mmol/kg diet DM intake) – Normalised concentration of $SCFAs_{terminal\ ileum\ digesta\ or\ faeces}$ (mmol/kg diet DM intake)

where $SCFAs_{blank\ initial}$ and $SCFAs_{blank\ final}$ are the SCFAs (mmol/kg DM) in the blanks prior to (initial) and after (final) *in vitro* fermentation, respectively. *In vitro* ileal and hindgut fermentation had their own blanks. $SCFAs_{jejunum\ or\ terminal\ ileum\ digesta}$ are the SCFAs (mmol/kg DM) in fresh terminal jejunal (ileal fermentation) or terminal ileal (hindgut fermentation) digesta, which represents the SCFAs present in the digesta before being fermented.

The calculation used to determine the normalised nutrient content was as follows:

10) Normalised nutrient $content_{terminal\ jejunal\ or\ terminal\ ileal\ digesta}$ (g/kg diet DM intake) = $nutrient\ concentration_{terminal\ jejunal\ or\ terminal\ ileal\ digesta}$ (g/kg DM) x ($TiO_{2\text{-diet}}/TiO_{2\text{-terminal jejunum or terminal ileal digesta}}$)

5.2.6. Statistical analysis

For this study, a sample size of five replicates was required to detect a statistical difference (5%) between GIT locations, with a power >80% at a two-tail 5% significance level based on variance (SD 2.4%) and means reported in previous studies [322, 324, 340].

The statistical analyses were performed in SAS version 9.4 (SAS Institute, Cary, NC, USA). The difference between ileal and hindgut fermentation within each pig for the OM fermentability, fermented OM, estimated synthesis of SCFAs, estimated disappearance of SCFAs, the normalised number of bacteria and archaea (total, phyla, and genus), and the normalised predicted metabolic activity was tested using a paired *t* test. The normal distribution of the difference for the *t* test was evaluated using the ODS graphics and the univariate procedure of SAS. Probability values of $P \leq 0.05$ were considered statistically significant, and a trend when $0.05 \leq P \leq 0.10$.

The non-parametric two-sample t-test (Monte Carlo permutation) from QIIME was used to determine alpha diversity significance. The Euclidean distance Principal Coordinates Analysis groupings were tested for significant separation using the Adonis test (9,999 permutations).

5.3. Results

All pigs were healthy except for a pig that had loose stools during the first experimental days.

5.3.1. In vitro *organic matter fermentability*

There was no difference ($P = 0.124$) between ileal and hindgut *in vitro* OM fermentability, 28 ± 1.9 and 35 ± 2.1 % (mean ± SEM), respectively (Figure 5.2). However, there was 66% more fermented OM in the ileum than in the hindgut ($P = 0.040$).

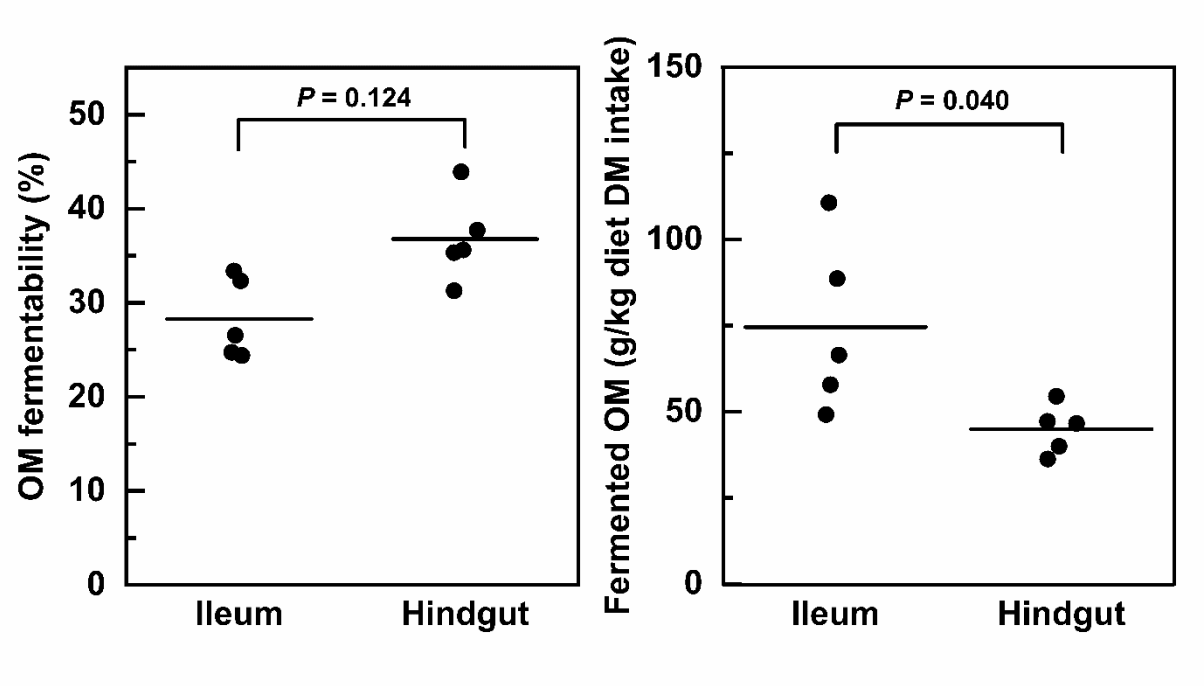

Figure 5.2: Ileal and hindgut *in vitro* OM fermentability values (left) and *in vivo/in vitro* fermented OM (right) of pigs fed a human-type diet (*n* = 5). The line for each GIT location represents the mean value. The effect of GIT location was tested using a paired *t* test. DM, dry matter, GIT, gastrointestinal tract; OM, organic matter.

5.3.1. Ileal and faecal microbiota

The normalised total number of bacteria and archaea in the caecum tended to be greater than in the ileum ($P = 0.09$; Table 5.1). Ileal digesta contained greater ($P \leq 0.05$) numbers of the family Micrococcaceae (42-fold greater), and the genera *Enterococcus* (32-fold greater), and *Leuconostoc* (55-fold greater). In contrast, caecal digesta had greater ($P \leq 0.05$) numbers of the class Clostridiales (57-fold greater), the families Coriobacteriaceae (91-fold greater), Lachnospiraceae (443-fold greater), and Tenericutes (24-fold greater), and the genera *Methanosphaera* (15-fold greater), *Blautia* (65-fold greater), *Coprococcus* (17-fold greater), *Ruminococcaceae* (227-fold greater), and *Ruminococcus* (82-fold greater).

Some bacteria were observed in considerable numbers in the caecal digesta (e.g., Bacteriodales, *Prevotella*, *Dorea*, *Lachnospira*, *Roseburia*, *Erysipelotrichaceae*, *Treponema*, and *TM7-3_F16*) were not detected in ileal digesta. Similar conclusions can be drawn from the relative abundance data (non-normalised data; Supplementary Figure 5.1 and Supplementary **Figure 5.2**). The alpha diversity tended to differ between the ileal and caecal microbiota (P = 0.09; Figure 5.3). Based on the normalised data, the microbiota in the caecal digesta resembled a more closely related community than the microbiota in the ileal digesta (Figure 5.4). The differences in the ileal and caecal microbiota composition are reflected in differences in their predicted metabolic activity according to PICRUSt analysis of pathways related to carbohydrate and protein metabolism (Supplementary Table 5.2). For example, the caecal predicted metabolic activity for pyruvate metabolism was 1.2-fold greater (P = 0.02) than the ileal pyruvate metabolism.

Table 5.1: Taxonomic composition in ileal and caecal digesta of pigs fed a human-type diet[1]

Phylum	Genus	GIT location		*P* value[2]
		Ileum	Caecum	
		x10^9 16S rRNA gene copy number/kg diet DM		
Total number of bacteria and archaea		828 ± 268	1946 ± 203	0.086
Actinobacteria		30.4 ± 0.81	32.0 ± 0.54	0.812
	Actinomycetales[3]	5.13 ± 3.14	0.08 ± 0.08	-
	Micrococcaceae[3]	32.5 ± 11.1	0.78 ± 0.38	0.046
	Coriobacteriaceae[3]	0.22 ± 0.13	20.4 ± 1.27	<0.001
	Collinsella	2.14 ± 2.07	10.2 ± 4.63	0.189
Bacteroidetes		0.10 ± 0.10	270 ± 66.0	-
	Bacteroidales[3]	0.05 ± 0.05	87.6 ± 19.7	-
	Prevotella	ND	175 ± 63.7	-
Euryarchaeota		1.17 ± 0.80	15.8 ± 3.07	0.011
	Methanosphaera	1.04 ± 0.83	15.3 ± 2.81	0.009
Firmicutes		784 ± 258	1513 ± 149	0.179
	Enterococcus	37.0 ± 12.1	1.15 ± 0.16	0.041
	Lactobacillus	10.6 ± 6.75	4.48 ± 1.33	0.342
	Leuconostoc	81.6 ± 29.0	1.48 ± 1.11	0.053
	Streptococcus	509 ± 160	299 ± 56.9	0.274
	Turicibacter	0.77 ± 0.37	15.6 ± 0.77	0.133
	Clostridiales[3]	3.46 ± 2.28	197 ± 22.6	0.001
	Christensenellaceae[3]	ND	0.45 ± 0.18	-
	Clostridiaceae[3]	30.7 ± 11.6	49.7 ± 14.9	0.626
	Lachnospiraceae[3]	0.18 ± 0.13	79.7 ± 12.9	<0.001
	Blautia	0.41 ± 0.34	26.8 ± 4.33	0.004
	Coprococcus	1.85 ± 1.71	30.9 ± 5.94	0.006
	Dorea	0.55 ± 0.51	22.3 ± 7.98	-
	Lachnospira	0.00 ± 0.00	40.1 ± 14.0	-
	Roseburia	0.02 ± 0.02	14.7 ± 10.6	-
	Ruminococcaceae	1.25 ± 1.01	284 ± 32.3	<0.001
	Ruminococcus	3.68 ± 3.20	302 ± 63.1	0.010
	Mogibacteriaceae	0.19 ± 0.05	1.60 ± 0.78	0.181
	Erysipelotrichaceae	0.18 ± 0.17	34.3 ± 12.2	-
Proteobacteria		7.34 ± 2.70	4.04 ± 1.24	0.420
	Enterobacteriaceae[3]	4.94 ± 1.69	1.99 ± 0.61	0.342
Spirochaetes		ND	47.7 ± 25.5	-
	Treponema	ND	47.3 ± 25.3	-
Tenericutes		0.60 ± 0.53	14.4 ± 3.84	0.017
TM7		0.11 ± 0.10	17.0 ± 9.75	-
	TM7-3_F16	ND	17.0 ± 9.75	-
Unassigned		9.08 ± 3.65	24.6 ± 6.21	0.147

[1] Values are means ± SEM; *n* = 5. Only bacteria phyla/genera with > 1% abundance in at least one of the samples are reported. GIT, gastrointestinal tract; ND, not detected.
[2] The statistical analysis was only conducted when bacteria were detected in a minimum of three pigs.
[3] Bacteria could only be classified as far as class, order, or family level.

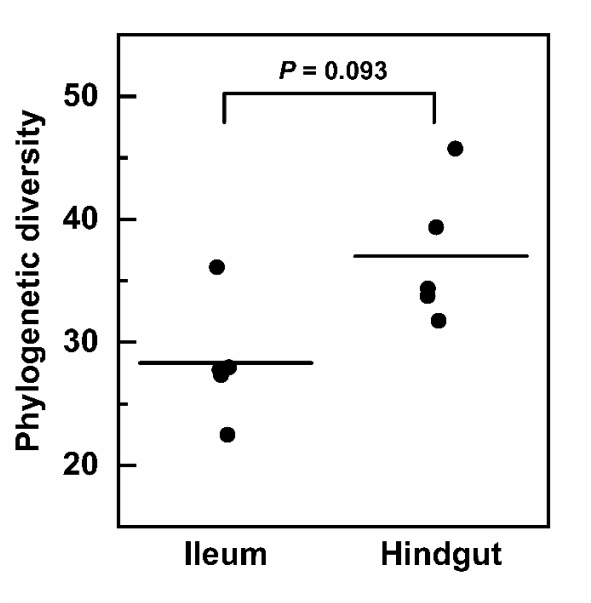

Figure 5.3: Alpha diversity numbers showing Faith's phylogenetic diversity of microbial communities in ileal and caecal digesta of pigs fed a human-type diet (*n* = 5) based on the normalised number of bacteria and archaea. The line for each GIT location represents the mean value. The effect of the GIT location was tested using a non-parametric two-sample *t* test. GIT, gastrointestinal tract.

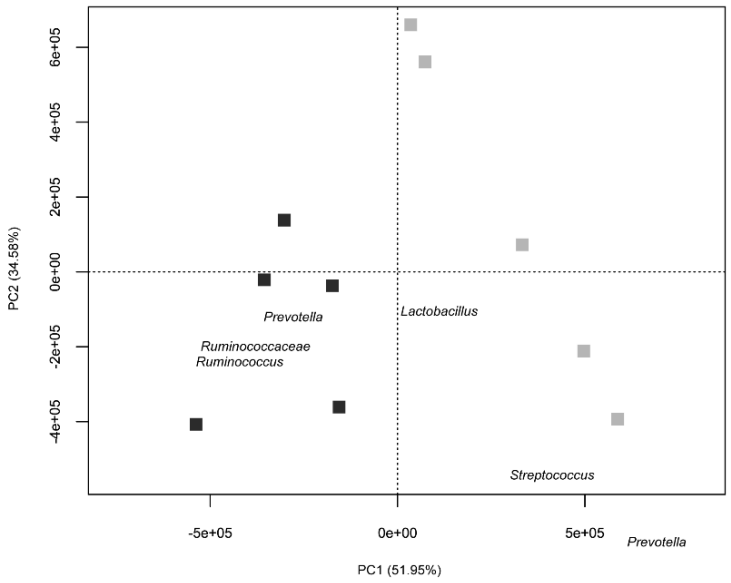

Figure 5.4: Euclidean Principal Coordinates Analysis of the distances of normalised relative abundance data (16S rRNA gene copy number/kg diet DM intake) in caecal (black squares) and ileal digesta (grey squares) for pigs fed a human-type diet (*n* = 5). Groupings exhibited a significant difference (*P* = 0.010, R^2 = 0.43) as determined by the non-parametric Adonis test (9,999 permutations). The most prevalent taxa responsible for variation in the plot are displayed. DM, dry matter.

5.3.1. Estimated ileal and hindgut short-chain fatty acid production

The estimated synthesis of acetate and iso-valerate were 6.1- and 1.3-fold greater (*P* ≤ 0.05), respectively, during ileal fermentation compared with hindgut fermentation (Figure 5.5). In contrast, the estimated synthesis of propionate, butyrate, and valerate was greater (4.6-, 8.3-, 4.5-fold, respectively; *P* ≤ 0.05) during hindgut fermentation compared with ileal fermentation. Similar trends were observed for the *in vitro* synthesis of SCFAs (Supplementary Table 5.3). Based on the estimated disappearance data (Figure 5.6), most

SCFAs disappeared in the GIT location where they were synthesised. The statistical differences were similar to those for the synthesis of SCFAs values.

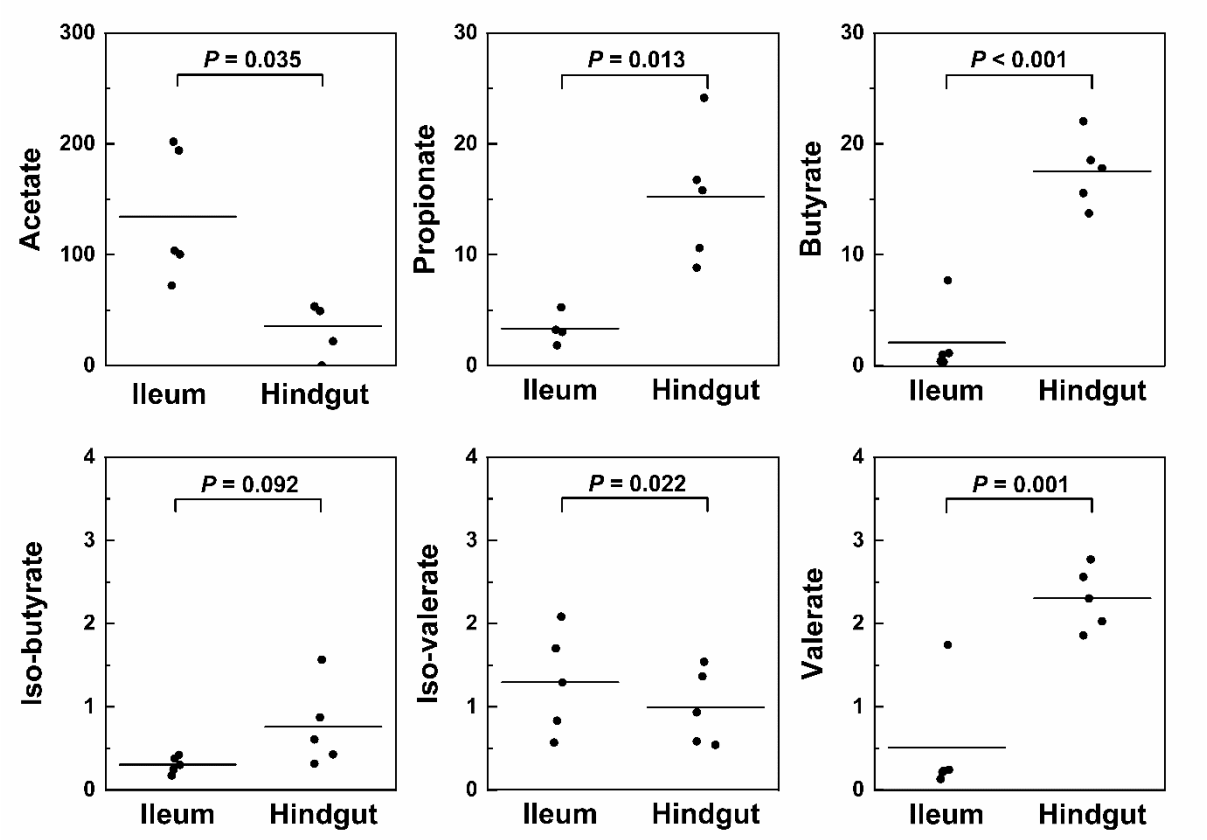

Figure 5.5: Estimated production of SCFAs (mmol/kg diet DM intake) in the ileum and hindgut of pigs fed a human-type diet (*n* = 5). The line for each GIT location represents the mean value. The effect of GIT location was tested using a paired *t* test. DM, dry matter, GIT, gastrointestinal tract, SCFA, short-chain fatty acid.

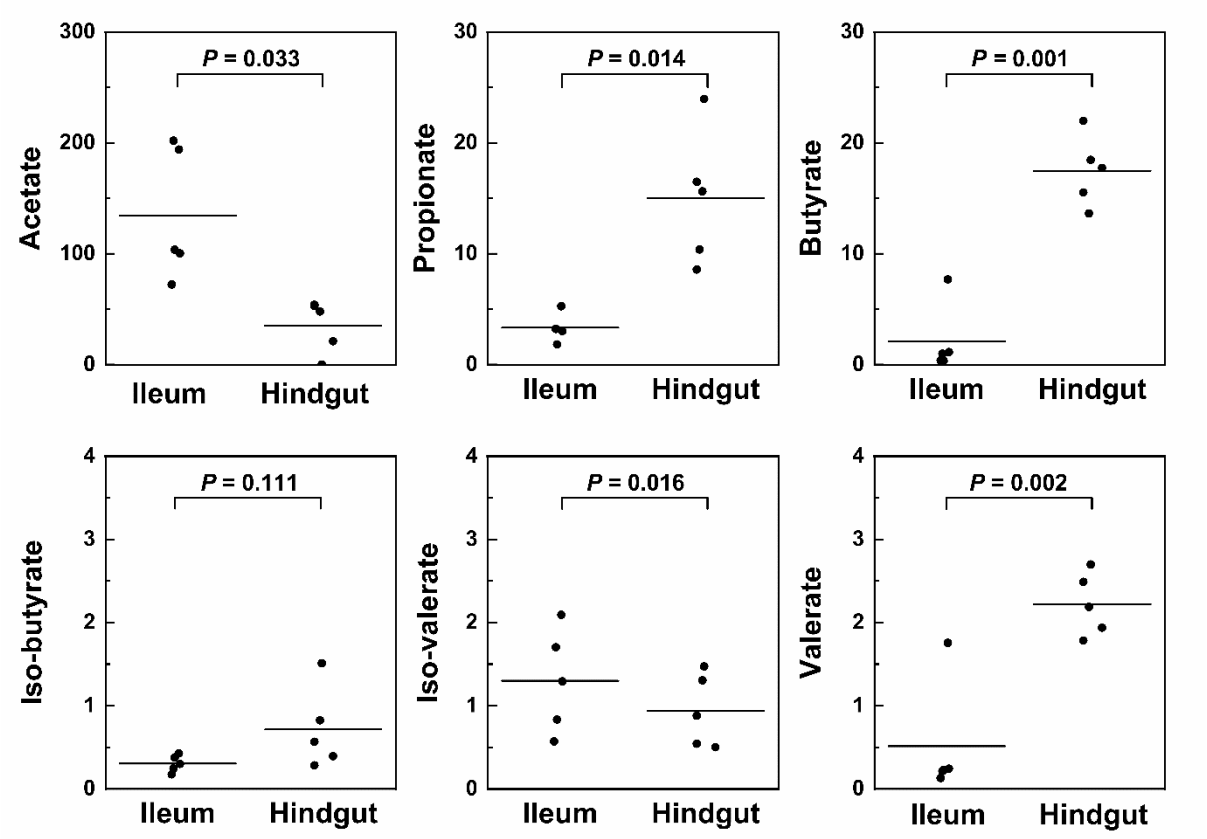

Figure 5.6: Estimated disappearance of SCFAs (mmol/kg diet DM intake) in the ileum and hindgut of pigs fed a human-type diet (*n* = 5). The line for each GIT location represents the mean value. The effect of GIT location was tested using a paired *t* test. DM, dry matter, GIT, gastrointestinal tract, SCFA, short-chain fatty acid.

5.4. Discussion

The ileal fermentation results confirm earlier observations in pigs [13, 323, 324] and establish that such fermentation is observed for a human-type diet. Indeed, one of the main results of this study was that the amount of fermented OM was 1.5-fold greater in the ileum compared with the hindgut, which is explained by a greater amount of OM entering the ileum than the hindgut (246 versus 141 g OM/kg diet DM intake) (Supplementary Table 5.4). A greater amount of acetate and iso-valerate were synthesised during ileal fermentation

compared with caecal fermentation, but for caecal fermentation, butyrate, propionate and valerate syntheses were greater. The current results are the first to demonstrate that SCFAs disappeared in the same GIT location where they were synthesised.

The normalised total number of bacteria and archaea (16S rRNA gene copy number/kg diet DM intake) in the caecal digesta ($19.5x10^{11}$) tended to be greater than in the ileal digesta ($8.3x10^{11}$). Rowan *et al.*, 1992 [341] reported that, when considering the dietary DM, the concentration of deoxyribonucleic acid (i.e., microbial marker) in fresh ileal digesta and faeces of pigs fed a human-type diet were not different. Recently, Montoya *et al.*, 2019 [322] reported two times greater normalised total number of bacteria (per kg diet DM intake) in ileal digesta compared with faeces of pigs fed diets containing kiwifruit as the sole dietary fibre source. Different conclusions are drawn when concentration data were normalised for diet DM intake, and the potential role of the ileal microbiota in fermenting undigested material is highlighted. In the pig ileal digesta, the predominant bacterial genus was *Streptococcus*, which is also the predominant bacterial genus in ileal effluent from human ileostomates [12]. *Streptococcus* is well adapted to the ileum because it can rapidly ferment simple carbohydrates (i.e., mono-, di- and oligosaccharides), which is important in the ileum as the retention time is shorter than in the hindgut [12, 326]. A greater number of *Streptococcus* was observed in the ileal digesta of the pigs fed the human-type diet compared with caecal digesta. This may be related to a tendency (P = 0.07) for a greater amount of starch, a rapidly fermentable carbohydrate, entering the ileum (31 g/kg diet DM intake) compared with the hindgut (14 g/kg diet DM intake) (Supplementary Table 5.4). The main bacteria in caecal digesta belonged to the class Clostridiales and the genera *Ruminococcus*, *Ruminococcaceae*, *Streptococcus,* and *Prevotella*. Both *Clostridium* sp. and *Ruminococcus* sp. have the ability to ferment cellulose [342]. This fibre needs a longer fermentation and is expected to be the main component of the insoluble dietary fibre fraction of the human-type

diet (Supplementary Table 5.1). The predicted metabolic activity related to carbohydrate and protein metabolism demonstrated that the ileal and caecal microbiota of the pigs fed the human-type diet had different metabolic activity profiles. The overall ileal predicted metabolic activity was 84% of the overall caecal predicted metabolic activity. Despite the differences in predicted metabolic activity, similar degrees of OM fermentability were observed during ileal and hindgut fermentation. The tendency towards greater microbial diversity in caecal digesta compared with ileal digesta coincides with a longer transit time in the hindgut, which gives bacteria a greater opportunity to grow, and for cross-feeding to occur [343]. The differences in the microbial community indicate that the ileal microbial community has evolved aligned to ferment rapidly fermentable substrates in accordance with the faster transit time. In contrast, the hindgut microbial community has evolved to ferment more slowly fermentable substrates in line with the slower transit time. The co-existence of these microbial communities may result in more efficient and effective fermentation of diets as humans eat diets that are complex in nature and composition.

During ileal fermentation, acetate was the main SCFA synthesised. In contrast, butyrate and propionate were synthesised in greater amounts during hindgut fermentation. Similar trends were reported for concentrations of these SCFAs in the ileal and caecal digesta of adult humans suffering sudden death [23]. However, concentration data need to be interpreted carefully as a concentration of SCFAs represents only the amount of SCFAs that has not been absorbed at the time of collection. Synthesis data are more meaningful. The differences seen here in the synthesis of SCFAs related to the ileal and hindgut fermentation may be related to several factors: (i) the incubation time of ileal fermentation (2 hours) compared with hindgut fermentation (24 hours), (ii) the microbial composition and (iii) the amount and chemical composition of the substrate available. A longer fermentation time may be one of the factors explaining the greater butyric acid synthesis. Longer fermentation

time allows cross-feeding to occur, whereby SCFAs like acetate can be converted into other SCFAs, like butyrate [42, 75, 344]. This would lower the concentration of acetate while increasing the concentration of butyrate. Another factor explaining the greater hindgut butyrate synthesis is the higher numbers of butyrate-synthesising bacteria reported in caecal digesta. Similarly, the greater caecal propionate synthesis may be related to higher numbers of propionate-synthesizing bacteria such as *Prevotella* [60] reported in the caecal digesta. The tendency to have lower phylogenetic diversity in the ileum compared with the caecum may explain the greater concentration of acetate observed after ileal fermentation compared with hindgut fermentation. Almost all GIT bacteria can synthesize acetate, whereas butyrate and propionate synthesis pathways are highly conserved in a limited amount of GIT bacteria [102]. The amount of crude protein entering the ileum was two-fold greater than that entering the hindgut (Supplementary Table 5.4), which may explain the greater iso-valerate synthesis during ileal fermentation since iso-valerate is a product of protein fermentation [24]. The SCFAs synthesised during ileal fermentation are expected to have similar effects on the ileal microbiota as reported for the caecal microbiota. For example, the synthesis of SCFAs reduces the pH and promotes the growth of different bacteria, like *Roseburia* [344].

Based on the estimated disappearance of SCFAs, the SCFAs were absorbed or metabolised in the same GIT location as they were synthesised. Previously, human studies have shown that SCFAs can be absorbed in both the ileum and hindgut [345, 346]. These results indicate that SCFAs synthesised in the ileum may have a local effect at the ileal epithelium or be absorbed to serve systemically in the host. For example, human ileal epithelial cells contain free fatty acid receptors, which, upon binding with SCFAs, can stimulate the production of satiety hormones, such as PYY and GLP-1 [347], which then increase ileal motility [206]. Dietary intervention may be a strategy to modulate the ileal synthesis of SCFAs and, therefore, deliver SCFAs both locally and systemically. Further

studies to investigate the influence of diet on ileal microbiota, their fermentation capacity and synthesis of SCFAs, and how ileal fermentation affects the host are warranted.

To compare estimated ileal and hindgut fermentation in the present work, a combined *in vivo*/*in vitro* methodology was used. The limitations and advantages of this methodology have been described previously [13]. One of the limitations of this *in vivo*/*in vitro* methodology is that *in vivo* absorption and fermentation of dietary and non-dietary nutrients occur simultaneously in the ileum. During *in vitro* fermentation, this absorption is not simulated. The presently described study involves only one human-type diet, and the work needs to be extended to more diverse diets and sources of dietary fibre. That the human-type diet was highly fermentable in the ileum, however, demonstrates that ileal fermentability may be a hitherto largely under-recognised yet important characteristic of foods and diets consumed by humans. Some studies have reported important ileal digestibility values of dietary fibre in human ileostomates [7, 145]. Both the ileal and hindgut fermentation of foods need to be better understood. However, applying the combined in vivo/in vitro methodology in humans requires an animal model to allow for the sampling of terminal jejunal digesta and ileal digesta. A faecal inoculum sourced from adult humans can replace the caecal inoculum used here to determine hindgut fermentation [284]. The growing pig is a valid animal model for the adult human for the foregut digestion of food [14, 328]. It can be expected that pig terminal ileal digesta samples can be used to provide a suitable substrate for the hindgut fermentation methodology (faecal inoculum). Porcine ileal digesta have been found to provide a suitable inoculum for a fermentation assay related to human ileal fermentation with similar OM fermentabilities of fibre substrates, regardless of differences in microbiota between the two species (Chapter 4).

5.5. Conclusion

In conclusion, the results of this experiment demonstrate that a diet consisting of foods commonly consumed by humans was well-fermented in the ileum of the growing pig. Indeed, the amount of fermented OM was greater during ileal than hindgut fermentation. The ileal fermentation synthesised an important amount of SCFAs (mainly acetic acid), which were absorbed or metabolised in the ileum.

Chapter 6: Type of dietary fibre is associated with changes in ileal and hindgut microbial communities in the growing pigs and influences *in vitro* ileal and hindgut fermentation

This chapter has been published in the Journal of Nutrition:

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Author's contribution: AME Hoogeveen was responsible for the SCFA (with technical assistance) and microbial analysis. She also performed the data analysis, bioinformatics, statistical analysis (under the guidance of Carlos Montoya) and wrote the draft manuscript.

Abstract

Background. The degree of ileal organic matter (OM) fermentation appears comparable to hindgut fermentation in growing pigs.

Objective. This study aimed to determine if dietary fibre sources with known different total gastrointestinal tract (GIT) fermentability in humans affect ileal and hindgut microbial communities and ileal fermentation in growing pigs used as an animal model for human adults.

Methods. Male pigs (21 kg bodyweight; nine-week-old; PIC Camborough 46 x PIC boar 356L; *n* = 8/diet) were fed for 42 days a diet containing cellulose (CEL, low fermentability)

as the sole fibre source (4.5%) or diets where half of the CEL was replaced by moderately fermentable fibre, psyllium (PSY) or kiwifruit (KF) fibre. For each diet, terminal jejunal (substrate) and ileal (inoculum) digesta were collected from the euthanised animal for *in vitro* ileal fermentation (2 h). Terminal ileal (substrate) and caecal (inoculum) digesta were used for *in vitro* hindgut fermentation (24 h). After *in vitro* fermentation, OM fermentation and short-chain fatty acid (SCFA) production were determined. Ileal digesta and faeces were collected for microbial analysis. Data were analysed by two-way ANOVA (diet x GIT region).

Results. In vitro ileal OM fermentation was, on average, 22% and comparable to hindgut OM fermentation. Ileal and hindgut OM fermentation, SCFA production, and microbial communities changed ($P \leq 0.05$) when CEL was partially replaced by KF or PSY. For instance, pigs fed the PSY diet had a threefold higher ($P \leq 0.05$) number of ileal and faecal bacteria than pigs fed the CEL and KF diets. Pigs fed the CEL diet had 4- higher ($P \leq 0.05$) hindgut valeric acid production than pigs fed the other diets.

Conclusion. Ileal fermentation is quantitatively significant. Partial substitution of CEL with more fermentable fibres influences both ileal and hindgut microbial communities and their fermentative capacity in growing pigs.

6.1. Introduction

The ileum (approximately the distal one-third of the small intestine) appears to be an important site of microbial fermentation in humans and monogastric animals [12, 20, 145, 154, 348, 349]. Such a conclusion, however, is mainly based on observations stemming from the density of microbes in the ileum [12, 20], ileal short-chain fatty acid (SCFA) concentrations [154, 348], and the disappearance of dietary fibres before the hindgut [145, 349]. A study using a combined *in vivo/in vitro* ileal fermentation assay has recently provided

direct evidence for the quantitative importance of ileal fermentation versus hindgut fermentation in the growing pig fed a human-type diet [286]. A similar degree of organic matter (OM) fermentation was observed in the ileum (28%) and the hindgut (35%) [286], with greater ileal production of acetic acid but lower production of propionic and butyric acids compared to the hindgut. The latter finding relates to a single mixed diet, and the generality of the finding has not yet been established. The quantitative importance of ileal fermentation for fibre sources differing in their total gastrointestinal tract (GIT) fermentability remains to be determined.

It is well established that different fibre sources have different GIT luminal properties, such as solubility and viscosity, which may influence the GIT microbial communities [311, 350-352], microbial metabolite production [30, 352, 353], and fermentation [30, 353]. For instance, cellulose (CEL; an insoluble fibre) has a low water-holding capacity, low viscosity, and low total GIT fermentability. In contrast, psyllium (PSY), a soluble fibre, has a high water-holding capacity, high viscosity and is moderately fermented in the total GIT of the adult human [30, 353]. Green kiwifruit (KF, *Actinidia deliciosa*, Hayward) has a 1:1 to 1:2 ratio of soluble:insoluble fibre [354, 355]) and a high water-holding capacity and high viscosity, and is extensively fermented in the GIT [349, 351, 352, 356]. Such differences in fermentability appear to be related to changes in microbial composition. For example, in growing pigs, intake of KF for seven days resulted in an increased number of total bacteria and *Bacteroides* in the hindgut, with a reduced number of *Escherichia coli* compared to a fibre-free or CEL-containing diet [351].

This study aimed to assess the quantitative importance of ileal fermentation (OM fermentation and SCFA production) and to investigate the effect of partial substitution of CEL (low total GIT fermentability) by dietary fibres, such as KF fibre and PSY, that are known to be more fermentable over the entire GIT on the ileal microbial community and

fermentation. A comparison was made with hindgut fermentation. It is hypothesised that the ileal microbial community, OM fermentation, and SCFA production differ when CEL is partially replaced by KF and PSY. In addition, despite differences in the microbial community between ileal and caecal digesta, it is hypothesised that the ileal OM fermentability is similar to the hindgut fermentability based on OM disappearance.

Considering the histological and physiological similarities from the mouth to the terminal ileum between the growing pig and the adult human [14, 357], the pig was used here as an animal model for human ileal fermentation. Ileal fermentation was compared with hindgut fermentation within the pig itself. The pig was not used as a model of human hindgut fermentation due to anatomical differences between the species in this respect.

6.2. Materials and methods

6.2.1. *Diets*

This study was part of a larger study that studied the effect of the KF, CEL and PSY on the histology of the epithelium and mucosa in the GIT [358]. The dietary fibre sources were: CEL (Avicel PH101, Hawkins Watts); KF fibre (provided as fresh KF; *Actinidia deliciosa* cv. Hayward); and PSY (95% purity, PSYH80, Davis Food Ingredients). Three semi-synthetic iso-fibre (45 g fibre/kg dry matter, DM) diets were formulated to provide similar macronutrient content and to meet the nutrient requirements of the growing pig as prescribed by the National Research Council [330] (Supplementary Table 6.1). One diet contained CEL as the sole fibre source, while in the other two diets, half of the CEL was replaced (on an equal fibre basis) by either KF fibre or PSY. For the KF diet, the ripe KF (firmness at 0.5-0.8 kg force) was peeled and crushed before mixing into the semi-synthetic diet before feeding. Titanium dioxide (TiO_2; 3 g/kg DM) was included in each diet as an indigestible marker.

6.2.2. In vivo *assay*

Animals and experimental design. The Massey University Animal Ethics Committee granted ethical approval for this study (Protocol 15/112). Twenty-four entire male pigs (PIC Camborough 46 x PIC boar 356L; 21.4 ± 1.2 kg bodyweight (BW), mean ± SD) were housed individually in metabolism crates (1.5 x 1.5 m) in a room maintained at 22 ± 2°C with a 12 h/12 h light/dark cycle. Pigs were randomly allocated to the three diets (n = 8). The experimental period was 42 days, which included a four-day adaptation period. The daily ration, adjusted weekly, was 100 g DM/kg of metabolic BW ($BW^{0.75}$) and fed three equal meals at 0800, 1200, and 1600 h. Pigs had free access to water. On day forty-two, pigs were fed hourly their daily ration as nine equal meals. Pigs were sedated and euthanised between 5 and 7 h after the first meal [13]. Digesta samples were collected as described previously [286]. Briefly, the small intestine was divided into three equal lengths. Digesta from the last 50 cm of the second (terminal jejunum) and last (terminal ileum) thirds were collected as substrates for the *in vitro* ileal and hindgut fermentation assays, respectively. From the remaining last third of the small intestine and the caecum, digesta were collected to prepare microbial inocula for the *in vitro* ileal and hindgut fermentation assays, respectively. To preserve anaerobic conditions, digesta samples were collected using plastic bags flushed with oxygen-free CO_2, after which digesta samples were stored at 4°C before *in vitro* fermentation. For microbial analysis, representative samples of ileal digesta and faeces were collected and stored at -80°C. Representative samples of terminal jejunal and terminal ileal digesta and faeces were collected, stored at -20°C, and freeze-dried before determining TiO_2 and nutrient composition.

6.2.3. In vitro *fermentation assays*

The *in vitro* ileal and hindgut fermentation assays have been described in detail previously and optimised for parameters such as pH, fermentation time, and inoculum concentration [13, 284]. For instance, these assays recommend 2 h and 24 h for ileal and hindgut fermentation, respectively. For the *in vitro* ileal fermentation assay, an ileal inoculum was prepared for each diet by mixing 220 g pooled ileal digesta obtained from pigs fed the respective diet with one litre sterilised anaerobic 0.1 M PBS (4.1 mM L-cysteine, pH 7) at 37°C [13]. An aliquot (5 mL) of ileal inoculum was added to CO_2-flushed bottles containing 5 mL of PBS either alone (blanks) or with fresh terminal jejunal digesta (1 g, substrate) obtained from pigs fed the respective diet. Six bottles were used per blank or substrate. Ileal fermentation took place anaerobically at 37°C for 2 h.

For the *in vitro* hindgut fermentation, 320 g pooled caecal digesta of pigs fed the respective diet were used to prepare a caecal inoculum for each diet. Caecal digesta were used to prepare the inoculum for hindgut fermentation, as in the hindgut, the caecal microbiota is the first to encounter the terminal ileal digesta. Terminal ileal digesta (1 g, substrate) of pigs fed the respective diet was fermented with the caecal inoculum at 37°C for 24 h under anaerobic conditions. Six bottles were used per blank or substrate. For some pigs, insufficient digesta were collected to perform *in vitro* fermentation, microbial and chemical analysis. Thus the number of observations per mean differ.

6.2.4. Chemical analysis

DM, ash [300], OM (DM – ash), starch (Kit AA/AMG, Megazyme), crude protein (nitrogen x 6.25; using an elemental analyser LECO), and TiO_2 [301] were determined for the diets and digesta. Diets were analysed for total lipids (using a Soxhlet extractor and petroleum ether

extraction), gross energy (using a LECO AC-350 Automatic Calorimeter), and total dietary fibre [331]. After *in vitro* ileal and hindgut fermentation, DM and ash contents were determined in half the samples (i.e., n = 3) [300]. Acetic, propionic, butyric, valeric, iso-butyric, and iso-valeric acids were quantified in the other half of the samples (i.e., n = 3) using gas chromatography as described previously [292] with iso-caproic acid as an internal standard.

6.2.5. Microbial analysis

DNA extraction. DNA from ileal digesta and faeces was extracted using a DNeasy PowerSoil kit (QIAGEN) according to the manufacturer's instructions with minor alterations. Samples were homogenised using a FastPrep FP120 Cell homogenizer (Thermo Fisher Scientific; three times for 60 seconds, 5.5 m/s, 5 minutes rest on ice between). The quantification and quality check of the extracted DNA were performed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A quantitative PCR was performed to determine the concentration of total bacteria, and taxonomic composition was determined by 16S rRNA gene sequencing (Supplemental methods).

6.2.6. Calculations

The indigestible marker TiO_2 was used to normalize all measures for the intake of DM (i.e., results are expressed per kg diet DM intake, Supplemental methods) [286].

6.2.7. Statistical analysis

Based on reported comparable means and variances, six replicates were found to be needed to achieve a significant difference (5% and 2.5 standard deviations) between diets with a power >80% at a two-tail 5% significance level [351]. In this study, eight replicates were used per diet to ensure that at least six pigs per diet completed the study period (42 days) and provided sufficient digesta per location for the *in vitro* fermentation assays and the required chemical and microbial analysis.

SAS version 9.4 (SAS Institute Inc.) was used to perform the statistical analyses. A two-way ANOVA model (diet x GIT region) was used with diet, GIT region, and diet x GIT region as fixed effects and with the pig as the experimental unit, using the Proc Mixed procedure. Repeated measures analysis could not be conducted for some pigs and GIT regions either because digesta were unavailable or the amount was insufficient to perform *in vitro* fermentation, microbial, and chemical analysis.

For the microbial data, only taxa present with >1% relative abundance in at least one sample were included in the statistical analyses. The microbial results showed that several bacterial taxa were either not present or present in only a few pigs fed the same diet. To consider this effect, a frequency analysis was first performed using a binary logistic regression for each GIT region using the Proc Glimmix procedure with 0 when the taxon was not present and 1 when a taxon had at least one read (i.e., relative abundance 0.001%).

For the taxonomic composition (i.e., number of gene copies per DM intake), initially, an analysis was conducted for the same taxa as for the frequency analysis. However, after considering the frequency analysis results, only taxa with >25% frequency of occurrence (i.e., $n \geq 2$) in ileal digesta or faecal samples across all diets were reported. This criterion was selected to remove any potential bias from taxa with a low frequency of occurrence. It

is important to highlight that for taxa with a frequency of occurrence lower than 25%, their frequency analysis and taxonomic composition analysis had similar trends across diets (data not shown). When a taxon was present in only one GIT region, a one-way ANOVA was performed to determine the effect of diet. A two-way ANOVA was performed when a taxon was present in both GIT regions. For the two-way ANOVA analysis, the interaction effect was removed from the final model when it was not significant ($P > 0.05$). The Bray-Curtis similarity Principal Coordinates Analysis (PCoA) groupings were tested for significant separation using the Adonis permutational test (999 permutations).

The model diagnostics for each response variable were tested using the ODS Graphics of SAS. The taxonomic composition data underwent a $\log_{10}$ transformation to achieve homogenous variance. The selected model for each response variable had homogenous variances across treatments. When the F-value of the model was significant ($P \leq 0.05$), the means were compared using the adjusted Tukey test. Probability values of $P \leq 0.05$ were considered statistically different, and $0.05 \leq P \leq 0.10$ were considered a trend.

6.3. Results

6.3.1. Ileal and hindgut organic matter fermentation

Ileal OM fermentation was quantitatively significant and was similar ($P = 0.44$) to the hindgut fermentation (22 ± 2.7 and 26 ± 2.9%, respectively, Figure 6.1). The ileal OM fermentation for pigs fed the KF diet tended ($P = 0.07$) to be 2.8-fold greater than for pigs fed the CEL diet. In contrast, the hindgut OM fermentation for pigs fed the PSY diet was 6.1- and 3.1-fold greater ($P \leq 0.05$) than for pigs fed the CEL and KF diets, respectively. For pigs fed the PSY diet, the hindgut OM fermentation was 2.5-fold higher ($P \leq 0.05$) than its ileal

counterpart. The *in vivo* ileal and hindgut OM fermentation followed similar trends (Supplemental Figure 6.1).

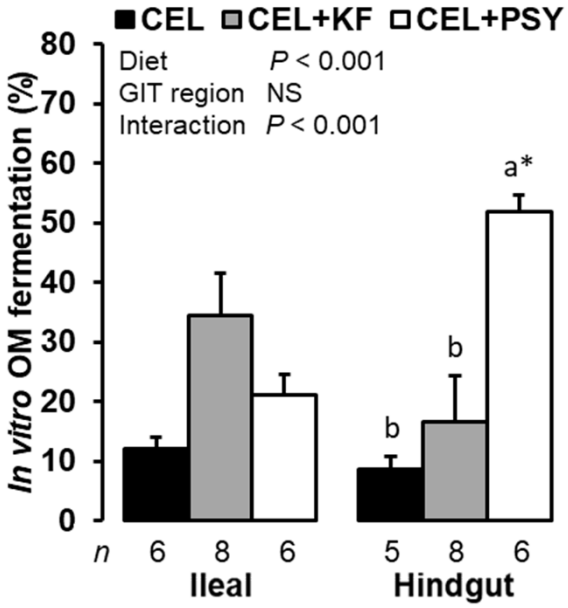

Figure 6.1: *In vitro* ileal and hindgut OM fermentation for pigs fed the CEL, KF, and PSY diets for 42 days. Values are mean ± SEM, *n* indicates the number of individual pigs. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. Means within each GIT region with a different letter differ ($P \leq 0.05$). Means with an asterisk for the hindgut differ ($P \leq 0.05$) from their ileal counterpart. CEL, cellulose; GIT, gastrointestinal tract; KF, kiwifruit; NS, not significant; OM, organic matter; PSY, psyllium.

6.3.2. Estimated ileal and hindgut short-chain fatty acid production

The estimated production of total SCFAs was 1.3-fold greater ($P \leq 0.05$, Figure 6.2) during ileal fermentation than during hindgut fermentation, irrespective of diet. Acetic acid production represented 95% of the total SCFAs produced in the ileum and 87% in the

hindgut (Figure 6.3). For pigs fed the CEL and PSY diets, a 2.0-fold greater production of total SCFAs was observed in both GIT regions than in pigs fed the KF diet. Similar results were found for acetic acid production. There was a significant interaction ($P \leq 0.05$) between diet and GIT region for the estimated production (combined *in vivo* and *in vitro* assays) of butyric, propionic, valeric, iso-butyric, and iso-valeric acids (Figure 6.3). Ileal production of these SCFAs was lower ($P \leq 0.05$) than their hindgut production, with a few exceptions. For instance, the ileal butyric acid production for pigs fed the KF diet was similar to the hindgut production. Pigs fed the PSY diet had a higher ($P \leq 0.05$) ileal production of butyric, propionic, valeric, and iso-butyric acids compared to the pigs fed the CEL diet. In the hindgut, pigs fed the CEL diet had a greater ($P \leq 0.05$) production of butyric, propionic, and iso-valeric acids than those fed the KF diet. The *in vitro* SCFA concentration data (i.e., non-normalised data) followed similar trends as those described above (Supplemental Figure 6.2 and Supplemental Figure 6.3).

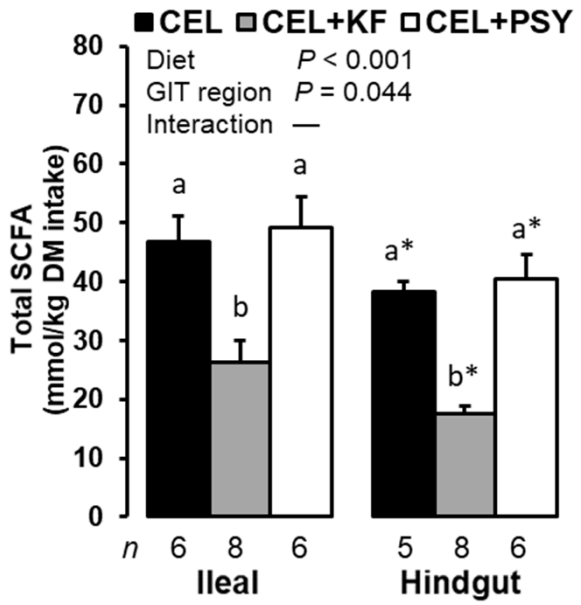

Figure 6.2: Estimated ileal and hindgut production of total SCFA for pigs fed the CEL, KF, and PSY diets for 42 days. Values are mean ± SEM, *n* indicates the number of individual pigs. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the

interaction between diet and GIT region. The interaction between diet and GIT region was not significant ($P > 0.05$) and was removed from the final model. Means with a different letter differ ($P \leq 0.05$). Means with an asterisk for the hindgut differ ($P \leq 0.05$) from their ileal counterparts. CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; NS, not significant; PSY, psyllium; SCFA, short-chain fatty acids.

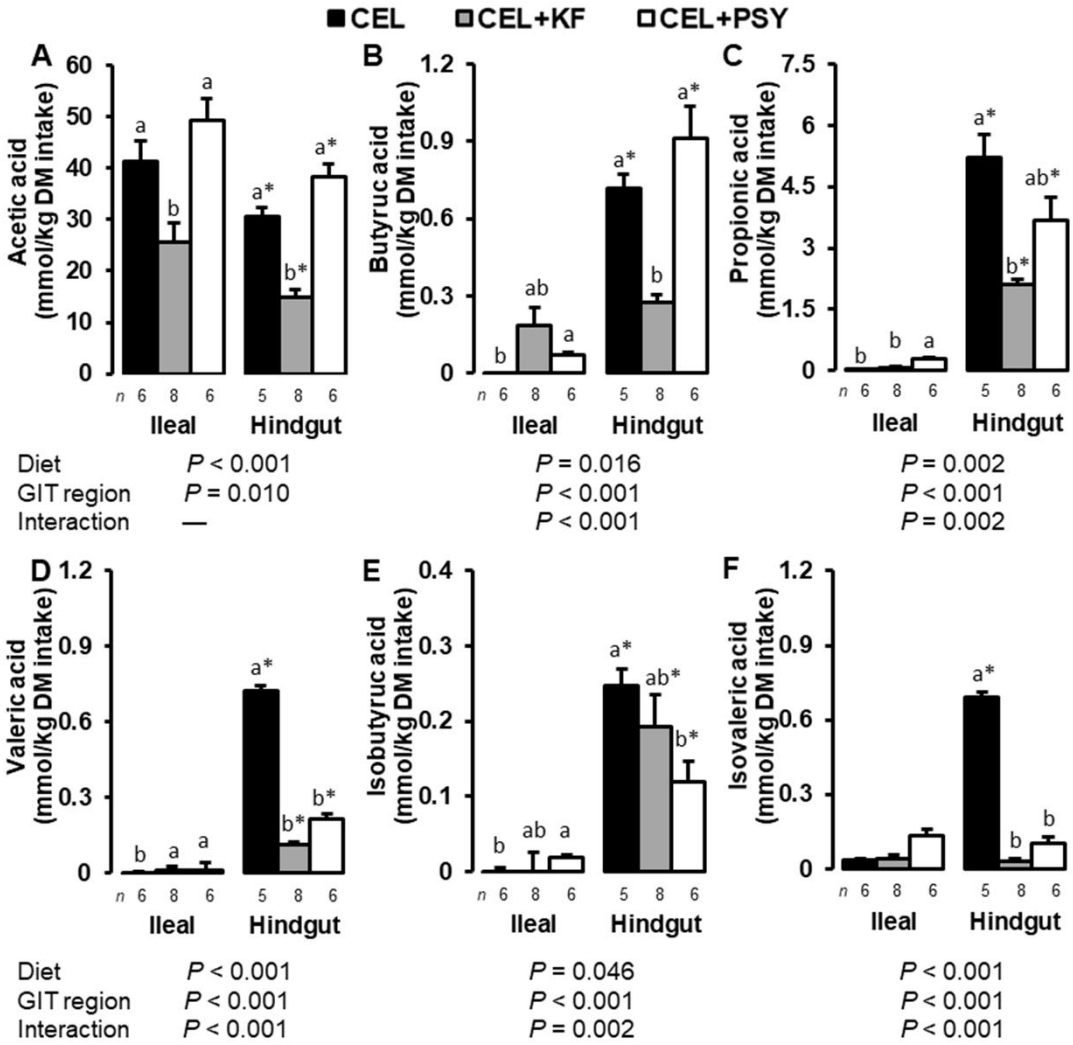

Figure 6.3: Estimated ileal and hindgut production of (A) acetic, (B) butyric, (C) propionic, (D) valeric, (E) iso-butyric, and (F) iso-valeric acids for pigs fed the CEL, KF, and PSY diets for 42 days). A two-way ANOVA model was used to assess the effect of diet, GIT region,

and the interaction between diet and GIT region. Values are mean ± SEM, *n* indicates the number of individual pigs. When the interaction between diet and GIT region was not significant ($P > 0.05$), it was removed from the final model. Means within each GIT region with a different letter differ ($P \leq 0.05$). Means with an asterisk for the hindgut differ ($P \leq 0.05$) from their ileal counterparts. When negative values were found (explained by corrected by SCFAs found in the blanks), values are assumed to be zero (i.e., the SCFA was not produced). CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; PSY, psyllium; SCFA, short-chain fatty acids.

6.3.3. Ileal and faecal microbiota

A total of 62,750 OTUs were identified with twenty-two unique bacterial phyla and 531 unique taxa across all samples. Of these taxa, 33% were solely found in ileal digesta, and 32% were unique to faeces. The ileal microbial community of pigs fed the KF diet had a greater ($P \leq 0.05$) α-diversity (i.e., Shannon index) compared to that of pigs fed the PSY diet (Figure 6.4). The α-diversity of the hindgut bacterial community was similar ($P > 0.05$) across diets but greater ($P \leq 0.05$) than for the ileal bacterial community. Based on β-diversity, the hindgut bacterial communities were more similar than the ileal community across all diets (Figure 6.5). In contrast, the ileal bacterial communities revealed a distinct separation between diets ($P \leq 0.05$).

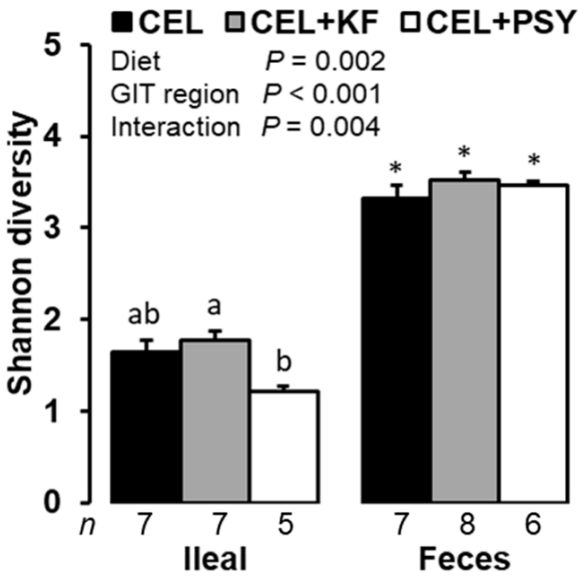

Figure 6.4: Shannon diversity of the microbial communities in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for 42 days, based on the data normalised for diet intake. Values are mean ± SEM, *n* indicates the number of individual pigs. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. Means within each GIT region with a different letter differ ($P \leq 0.05$). Means with an asterisk for the faeces differ ($P \leq 0.05$) from their ileal counterparts. CEL, cellulose; GIT, gastrointestinal tract; KF, kiwifruit; PSY, psyllium.

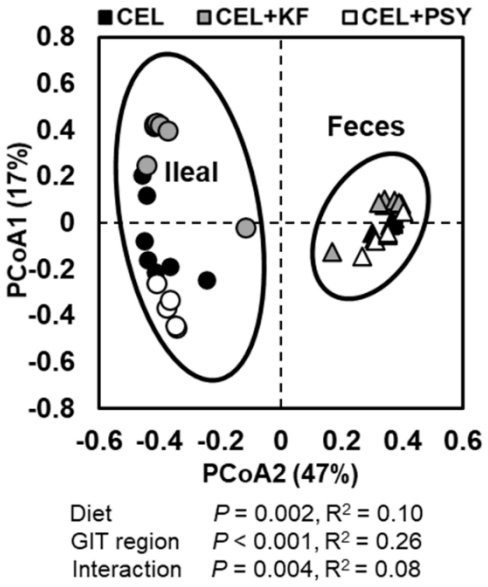

Figure 6.5: Bray-Curtis dissimilarity PCoA plot in ileal digesta and faeces of pigs given the CEL, KF, and PSY diets for 42 days based on the data normalised for diet intake. Every data point represents an individual sample, *n* = 5-8 pigs per diet and GIT region. The Adonis test (999 permutations) was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region on the groupings. CEL, cellulose; GIT, gastrointestinal tract; KF, kiwifruit; PCoA, principal coordinate analysis; PSY, psyllium.

The frequency of occurrence of several bacterial taxa differed ($P \leq 0.05$) across diets and GIT regions (Supplementary Table 6.2). For example, in ileal digesta, *Enorma* were only found in 14% and 57% of the pigs given the CEL and KF diet, respectively, and were not present in pigs given the PSY diet. *Enorma* were present in all faecal samples. Only taxa present in more than 25% (i.e., $n \geq 2$) of the ileal digesta or faecal samples across all diets were included in the statistical analysis of the taxonomic composition (Supplementary Table 6.3). Only taxa for which there was a significant ($P \leq 0.05$) diet effect or a significant interaction between diet and GIT region are shown in Table 6.1.

Table 6.1: Taxonomic composition in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for 42 days[1]

Phylum	Genus	Ileal digesta			SEM	Faeces			SEM	*P* value[2]		
		CEL	CEL +KF	CEL +PSY		CEL	CEL +KF	CEL +PSY		Diet	GIT region	Interaction
Sample size, *n*[3]		7	7	5		7	8	6				
		Log_{10} 16S rRNA gene copies/kg diet DM intake										
Total bacteria		13.2^{b}	13.1^{b}	13.6^{a}	0.177	12.8^{b}	12.8^{b}	13.3^{a}	0.112	0.007	0.057	-
Actinobacteria												
	Collinsella	ND	ND	ND	-	10.3^{b}	10.1^{b}	11.2^{a}	0.225	0.003	-	-
Bacteroidota		10.9^{b}	10.9^{b}	11.3^{a}	0.157	12.3^{b}*	12.3^{b}*	12.7^{a}*	0.117	0.019	<0.001	-
	Bacteroidales[4]	8.61^{b}	9.07^{ab}	9.49^{a}	0.234	10.3^{b}*	10.7^{ab}*	11.1^{a}*	0.177	0.044	<0.001	-
	Bacteroidia[4]	ND	ND	ND	-	9.34^{b}	9.71^{ab}	10.4^{a}	0.306	0.013	-	-
	Parabacteroides	8.52^{b}	8.95^{ab}	9.15^{a}	0.271	10.8^{b}*	11.3^{ab}*	11.5^{a}*	0.168	0.037	<0.001	-
	Prevotella	9.38^{ab}	8.62^{b}	9.72^{a}	0.291	11.0^{ab}*	10.2^{b}*	11.3^{a}*	0.286	0.045	<0.001	-
	Prevotellaceae NK3B31 group	8.09^{b}	8.51^{ab}	9.05^{a}	0.318	10.4^{b}*	10.8^{ab}*	11.4^{a}*	0.218	0.015	<0.001	-
	Prevotellaceae UCG001	ND	ND	ND	-	9.27^{ab}	8.96^{b}	10.6^{a}	0.371	0.013	-	-
	Tannerellaceae[4]	ND	ND	ND	-	9.97^{b}	10.5^{ab}	11.1^{a}	0.242	0.022	-	-
Desulfobacterota		9.41^{ab}	9.20^{b}	9.82^{a}	0.202	11.2^{ab}*	11.0^{b}*	11.6^{a}*	0.115	0.003	<0.001	-
Firmicutes		12.8^{b}	12.9^{ab}	13.1^{a}	0.144	12.5^{b}*	12.5^{ab}*	12.8^{a}*	0.101	0.042	0.010	-
	Acidaminococcus	7.76^{ab}	6.66^{b}	8.00^{a}	0.345	10.6^{ab}*	9.5^{b}*	10.9^{a}*	0.338	0.036	<0.001	-
	Anaerovoracaceae[4]	8.82^{a}	7.79^{b}	8.57^{ab}	0.284	10.1^{a}*	9.11^{b}*	9.89^{ab}*	0.277	0.009	<0.001	-
	Carnobacterium	11.5^{b}	11.6^{ab}	11.9^{a}	0.152	10.4^{b}*	10.5^{ab}*	10.8^{a}*	0.092	0.045	<0.001	-
	Catenisphaera	ND	ND	ND	-	10.7^{a}	9.01^{b}	10.1^{a}	0.212	<0.001	-	-
	Clostridium sensu stricto 1	9.34^{b}	11.2^{a}	10.3^{b}	0.310	8.91^{b}	10.8^{a}	9.84^{b}	0.302	<0.001	0.170	-
	Enterococcus	10.5^{b}	10.7^{b}	11.1^{a}	0.207	9.63^{b}*	9.83^{ab}*	10.2^{a}*	0.124	0.008	<0.001	-

Erysipelotrichaceae UCG006	ND	ND	ND	-	10.2^{a}	7.95^{b}	10.3^{a}	0.484	0.003	-	-
Erysipelotrichaceae UCG009	ND	ND	ND	-	9.55^{a}	7.64^{b}	9.55^{a}	0.474	0.011	-	-
Family XIII AD3011 group	7.87^{b}	8.72^{a}	8.81^{a}	0.209	10.5^{b}*	11.3^{a}*	11.4^{a}*	0.164	<0.001	<0.001	-
Holdemanella	ND	ND	ND	-	9.29^{b}	9.45^{ab}	10.19^{a}	0.300	0.009	-	-
Lachnospiraceae[4]	10.1^{ab}	9.75^{b}	10.2^{a}	0.181	11.8^{ab}*	11.4^{b}*	12.0^{a}*	0.118	0.012	<0.001	-
Lachnospiraceae NK3A20 group	ND	ND	ND	-	10.2^{a}	7.97^{b}	10.5^{a}	0.295	<0.001	-	-
Lactobacillus	12.1^{a}	11.4^{b}	12.2^{a}	0.197	10.5^{a}*	9.80^{b}*	10.7^{a}*	0.158	<0.001	<0.001	-
Lactococcus	12.7	12.7	12.2	0.234	10.2*	10.8*	10.7*	0.219	0.448	<0.001	0.023
Megasphaera	7.81	7.62	8.04	0.447	10.6^{a}*	8.81^{b}	11.4^{a}*	0.423	0.004	<0.001	0.039
Mitsuokella	ND	ND	ND	-	10.1^{a}	7.49^{b}	8.94^{b}	0.373	<0.001	-	-
Oscillospirales[4]	8.55^{b}	8.94^{ab}	8.94^{a}	0.192	11.3^{b}*	11.6^{ab}*	11.6^{a}*	0.146	0.016	<0.001	-
Oscillospiraceae UCG002	8.84^{b}	8.86^{ab}	9.32^{a}	0.233	10.6^{b}*	10.9^{ab}*	11.4^{a}*	0.228	0.007	<0.001	-
Phascolarctobacterium	8.10^{b}	8.24^{ab}	8.79^{a}	0.268	10.8^{b}*	10.9^{ab}*	11.5^{a}*	0.166	0.018	<0.001	-
Romboutsia	9.71^{b}	10.8^{a}	10.1^{ab}	0.259	8.98^{b}*	10.1^{a}*	9.37^{ab}*	0.252	0.0.03	0.007	-
Selenomonadaceae[4]	ND	ND	ND	-	10.8^{a}	9.23^{b}	10.2^{ab}	0.385	0.027	-	-
Weissella	11.6	11.9	11.3	0.235	8.58^{b}*	9.48^{a}*	9.62^{a}*	0.219	0.019	<0.001	0.004
Proteobacteria	12.8^{b}	12.4^{c}	13.4^{a}	0.188	11.7^{b}*	11.3^{c}	12.3^{a}*	0.117	<0.001	<0.001	-
Desulfovibrio	9.37^{ab}	9.14^{b}	9.76^{a}	0.202	11.2^{ab}*	10.9^{b}*	11.5^{a}*	0.115	0.003	<0.001	-
Enterobacterales[4]	10.8^{b}	10.5^{b}	11.4^{a}	0.189	9.54^{b}*	9.24^{b}*	10.1^{a}*	0.117	<0.001	<0.001	-
Escherichia-Shigella	12.7^{a}	11.3^{b}	13.3^{a}	0.208	11.2^{b}*	11.0^{b}	12.1^{a}*	0.197	<0.001	<0.001	0.013
Klebsiella	11.2^{ab}	10.5^{b}	12.2^{a}	0.374	9.92^{ab}*	9.15^{b}*	10.8^{a}*	0.296	0.002	<0.001	-
Succinivibrionaceae[4]	ND	ND	ND	-	9.30^{a}	7.51^{b}	9.36^{a}	0.399	0.004	-	-
Succinivibrio	ND	ND	ND	-	10.5^{ab}	9.58^{b}	11.2^{a}	0.300	0.005	-	-
Spirochaetota	7.56^{b}	8.61^{a}	8.39^{ab}	0.312	10.2^{b}*	11.2^{a}*	11.0^{ab}*	0.304	0.016	<0.001	-
Treponema	7.55^{b}	8.61^{a}	8.38^{ab}	0.311	10.2^{b}*	11.2^{a}*	11.0*	0.303	0.016	<0.001	-

Synergistota	7.68[b]	8.12[b]	9.19[a]	0.310	9.88[b]*	10.3[b]*	11.4[a]*	0.215	<0.001	<0.001	-
Cloacibacillus	ND	ND	ND	-	9.24[b]	9.77[b]	10.7[a]	0.248	0.003	-	-

[1] Values are means with pooled SEM per GIT region. Only taxa with >1% relative abundance in at least one of the samples and with >25% frequency in the ileal digesta or faecal samples (Supplementary Table 6.2) that showed a significant effect of diet are presented (Supplementary Table 6.3). Data were $\log_{10}$ transformed to achieve homogenous variance. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. Means within each gastrointestinal tract region with a different letter differ ($P < 0.05$), and the means with an asterisk for the faeces differ ($P \leq 0.05$) from their ileal counterparts. CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; ND, not detected; PSY, psyllium.
[2] When the interaction between the diet and GIT region ($P > 0.05$) was not significant, this interaction was removed from the model.
[3] *n* indicates the number of individual pigs. The different numbers of pigs are due to insufficient digesta or faeces collected for analysis.
[4] Bacteria could only be classified as far as order or family level.

Table 6.2: Nutrient composition and (normalised) nutrient contents of terminal jejunal and terminal ileal digesta of pigs fed the CEL, KF, and PSY diets for 42 days[1]

	Terminal jejunum				Terminal ileum				*P* value[2]		
	CEL	CEL+KF	CEL+PSY	SEM	CEL	CEL+KF	CEL+PSY	SEM	Diet	GIT region	Interaction
Sample size, *n*[3]	7	7	6		5	8	6				
Nutrient composition, g/kg DM substrate											
Organic matter	806	826	788	15.4	742*	762*	724*	15.7	0.143	<0.001	-
CP	153	136	119	12.8	65.9^{b*}	120^{a}	104^{a}	7.0	0.141	<0.001	0.002
Starch	130^{ab}	123^{b}	163^{a}	23.6	84.6^{ab*}	35.1^{b*}	68.0^{a*}	24.9	0.035	0.001	-
Normalised nutrient content, g/kg diet DM intake[4]											
Organic matter	163^{a}	130^{b}	151^{a}	7.28	93.5^{a*}	59.7^{b*}	81.4^{a*}	4.55	<0.001	<0.001	-
CP	25.3^{ab}	25.1^{b}	28.4^{a}	2.44	9.63^{ab*}	9.41^{b*}	12.8^{a*}	0.84	0.014	<0.001	-
Starch	29.2^{ab}	23.1^{b}	28.5^{a}	4.70	8.35^{ab*}	2.22^{b*}	7.60^{a*}	2.95	0.022	<0.001	-

[1] Values are means and pooled SEM per GIT region. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. The means within each gastrointestinal tract region with a different letter differ ($P \leq 0.05$), and the means with an asterisk in the terminal ileum differ ($P \leq 0.05$) from their terminal jejunal counterparts. CEL, cellulose; CP, crude protein; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; PSY, psyllium.
[2] When the interaction between the diet and GIT region was not significant ($P > 0.05$), this interaction was removed from the model.
[3] *n* indicates the number of individual pigs. The different numbers of pigs are due to insufficient digesta collected for analysis.
[4] Data was normalised for the DM intake using the ratio of the internal marker (i.e., titanium dioxide) in the diet versus digesta (Supplemental methods).

The normalised total number of bacteria (i.e., gene copy number/kg diet DM intake) tended (P = 0.06) to be 2.2-fold greater in ileal digesta compared to faeces across all diets (Table 6.1). On average, the total number of bacteria was 2.7- and 3.2-fold greater ($P \leq 0.05$) across both GIT regions for pigs fed the PSY diet compared to pigs fed the CEL and KF diets, respectively. A few bacterial taxa, like *Enterococcus*, were found in greater ($P \leq 0.05$) numbers in ileal digesta than faeces, irrespective of diet. However, most bacterial taxa were found in greater numbers in faeces than ileal digesta. The PSY diet increased ($P \leq 0.05$) the ileal and faecal number of gene copies for sixty-six percent of the taxa compared to the CEL or KF diet (Table 6.1). However, a 15-fold greater ($P \leq 0.05$) number of *Mitsuokella* was found in the faeces of pigs fed the CEL diet compared to the faeces of pigs fed the PSY diet, and a 9.3-fold greater ($P \leq 0.05$) number of *Clostridium sensu stricto 1* was found in the ileal digesta and faeces of pigs fed KF compared to pigs fed PSY. Similar trends were observed for both the relative abundance data (Supplemental Figure 6.4 and Supplemental **Figure 6.5**) and the predicted metabolic activity of ileal and faecal microbial communities (Supplementary Table 6.4).

6.3.4. Nutrient content of the terminal jejunal and terminal ileal substrates

Greater amounts of OM (1.9-fold), crude protein (2.5-fold), and starch (4.4-fold) entered the ileum compared to entering the hindgut across all diets (based on the normalised data, Table 6.2). Pigs fed the CEL and PSY diets had, on average across both GIT regions, 1.4- and 1.2-fold greater ($P \leq 0.05$) amounts of OM entering both the ileum and hindgut than pigs fed the KF diet, respectively. Pigs fed the PSY diet had ($P \leq 0.05$) (or tended to have (P = 0.06)) a greater amount of crude protein (KF and CEL, respectively) and starch (KF only) entering both GIT regions. For example, the pigs fed the PSY diet had (or tended to have), on

average across both GIT locations, 1.2-fold more crude protein entering both the ileum and hindgut of pigs compared to the KF ($P \leq 0.05$) and CEL ($P = 0.06$) diets.

6.4. Discussion

Based on earlier observations [282], this study confirms the hypothesis that ileal fermentation is quantitatively significant, with 22% fermentation on average. The degree of ileal fermentation was similar to hindgut fermentation, despite differences in microbial communities. Partially replacing fibre with low total GIT fermentability (CEL) with fibre with higher total GIT fermentability (KF and PSY) affected OM fermentation, microbial community composition, and SCFA production in both the ileum and hindgut.

Ileal fermentation

The tendency for a greater *in vitro* ileal OM fermentation for pigs fed the KF diet compared to pigs fed the CEL diet could be due to a high (80%) expected fermentability of the soluble fibre fraction of KF at the end of the small intestine of the growing pig [349]. CEL is slowly fermented [350] and, therefore, had a lower but still quantitatively important *in vitro* ileal OM fermentation.

The greater ileal production of acetic acid for pigs fed the PSY diet could be explained in that (i) the pigs fed the PSY diet had higher ileal numbers of acetic acid-producing bacteria, such as *Carnobacterium* and *Lactobacillus* (on average +110% and +120%, respectively); (ii) a greater amount of OM entering the ileum of the pigs fed the CEL and PSY diets compared to the pigs fed the KF diet; (iii) the chemical composition of the OM entering the small intestine. For instance, more starch (40 g more starch per kg DM diet intake) entered

the ileum of the pigs fed the PSY diet when compared to pigs fed the KF diet; (iv) structural differences across fibres (e.g., monosaccharide composition, branching, and degree of polymerization) that influence their degree of fermentation and production of SCFAs [359]; and (v) the effect of dietary fibres on endogenous losses [141, 360, 361]. Montoya *et al.*, 2017 [360] have shown that endogenous losses (e.g., mucin) are the major contributor to the total hindgut production of SCFA in growing pigs fed diets containing KF as the sole fibre source. These factors could also explain why pigs fed the PSY diet had higher ileal production of butyric (vs CEL), propionic (vs CEL and KF), valeric (vs CEL), and iso-butyric (vs CEL) acids. Though the diets were formulated to contain the same quantity of macronutrients, the determined total starch, crude protein, total sugar, and ash were slightly higher for the KF diet than for the PSY and CEL diets. As these dietary nutrients are highly digestible in the upper GIT (e.g., 93.9% of the starch was digested before the terminal jejunum), it was assumed that these minor differences did not affect the microbial community and their fermentation behaviour.

The higher α-diversity (i.e., number of taxa per sample) in the ileal microbial community of pigs fed the KF diet (vs PSY) could be because the KF contained multiple types of fibre (i.e., pectic polysaccharides, cellulose, and hemicellulose) [362]. Therefore, the KF diet offered a more diverse substrate for the ileal microbiota. A more diverse microbial community has been linked to greater stability, productivity, and resistance against pathogens [363]. The lower α-diversity in the ileal digesta of pigs fed the PSY diet is ascribed to specific bacteria (such as *Klebsiella* and *Prevotella*) being present in much greater numbers than in the ileal digesta of pigs fed the CEL and KF diets. In the ileal digesta of pigs fed the KF diet, higher numbers of *Clostridium sensu stricto 1* were found. *Clostridium sensu stricto 1* is a known butyric acid producer [364]. Their higher numbers explain why the pigs fed the KF diet had (or tended to have) greater butyric acid production in the ileum than pigs fed the CEL and

PSY diets. These results confirm previous reports of diet affecting the ileal microbial community in humans [20] and pigs [289]. The effects of diet on α-diversity were also reflected in the values of the β-diversity (i.e., variability in community composition between samples). Although the pigs used in this study were from the same breed and were raised in the same environment, some taxa were found to be present or absent in different individual pigs, even when receiving the same diet. This demonstrates that individualistic factors affect the microbial community, but the fermentation behaviour (based on OM fermentation and SCFA results) and predicted metabolic activity (according to PICRUSt) to a lesser extent.

Similar effects to the findings in this study may also be expected in the ileum of adult humans due to the inter-species similarities in the small intestine [14, 357] and thus deserve investigation. Considering that SCFAs produced in the ileum can be absorbed directly in this GIT region [206, 286, 345], it is expected that dietary fibres can affect host health, as shown for hindgut fermentation [2, 3]. The pig has been shown to be a valid model for ileal fermentation (OM fermentability and organic acid production) in adult humans, despite observed differences in the ileal microbial communities between the species (Chapter 4).

Hindgut fermentation

The pigs fed the PSY diet had greater *in vitro* hindgut OM fermentation than those fed the CEL and KF diets. This could be explained by KF fibre being highly fermented in the ileum. Eighty percent of the soluble fibre in KF is fermented before the terminal ileum [349]. Thus, in the terminal ileal substrate of the pigs fed the KF diet and of the pigs fed the CEL diet, CEL was likely the main (or only for the CEL diet) fermentable fibre during *in vitro* hindgut fermentation. CEL is a poorly fermentable fibre [350], which leads to low hindgut OM

fermentation. Generally, the pigs fed the KF diet had lower estimated hindgut production of SCFAs than those fed the CEL or PSY diets. Low butyric acid production could be related to (i) a lower amount of nutrients, especially starch, available in the substrate from pigs fed the KF diet compared to pigs fed the CEL and PSY diets (butyric acid production is positively correlated with the amount of starch in the substrate [322, 365]); (ii) the lower number of the butyric acid-producers *Erysipelotrichaceae UCG006 and UCG009*, and *Lachnospiraceae NK3A20 group* compared to pigs fed the CEL and PSY diets.

In the substrate for hindgut fermentation, nutrients other than fibre, such as starch and protein, were present, which can be utilised by the microbial community resulting in SCFAs. This could explain the higher butyric (KF only), propionic, valeric, and iso-valeric acid production in the pigs fed the CEL than in the pigs fed the KF and PSY diets. For example, the greater butyric acid production may have been due to the higher (but not significant) starch content in the substrate.

Ileal vs hindgut fermentation

The ileal microbial community was found to have a 9-fold greater predicted metabolic activity related to fermentation (i.e., carbohydrate and protein metabolism) than the faecal microbial community, which is supported by the observed total number of bacteria tending to be greater in the ileal digesta compared to the faeces. These results suggest that the ileal microbial community is adapted to a faster transit time by metabolizing rapidly fermentable substrates. In comparison, the hindgut microbial community is more adapted to a slower transit time. It is specialised in metabolizing slowly fermentable substrates, as suggested elsewhere [12, 20]. The greater metabolic activity of the ileal microbiota can explain the

similar *in vitro* ileal and hindgut OM fermentation values observed for the CEL and KF diets. This confirms previous findings for pigs fed a human-type diet [286].

In general, more acetic acid was produced during ileal fermentation. In contrast, lower butyric, propionic, valeric, and iso-butyric acids were produced. Similar trends have been reported for pigs fed a human-type diet [286] and during *in vitro* fermentation of purified fibre substrates [267]. A greater ileal acetic acid production is likely related to the higher ileal numbers of acetic acid producers (e.g., *Carnobacterium*, *Cronobacter*, *Escherichia-Shigella*, *Lactobacillus*, *Romboutsia,* and *Weissella*), while to lower productions of butyric, propionic, valeric, and iso-butyric acids are likely related to the lower numbers of butyric and propionic acid-producers (e.g., *Acidaminococcus*, Lachnospiraceae, *Megasphaera*, *Ruminococcus*, *Selenomonas*, *Subdoligranulum*, and *Succiniclasticum*).

6.5. Conclusion

In conclusion, ileal OM fermentation is quantitatively important and similar to hindgut OM fermentation, even though the microbial communities were dissimilar. The ileal microbial community and its fermentation patterns (based on OM fermentation and SCFA production) are influenced by partially replacing a low fermentable fibre (CEL) in the diet of growing pigs with more fermentable fibres (KF and PSY). Digesta of pigs fed dietary fibre sources are fermented to different degrees in different parts of the GIT. When evaluating the influence of dietary fibre on GIT fermentation, ileal fermentation needs to be considered, in addition to hindgut fermentation.

Chapter 7: *In vitro* ileal fermentation was affected more by the fibre source fermented than the ileal microbial community of growing pigs

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Hoogeveen AME, Moughan PJ, Hodgkinson SM, Stroebinger N, Yu W, Rettedal EA, McNabb WC, Montoya CA. *In vitro* ileal fermentation was affected more by the fibre source fermented than the ileal microbial community of growing pigs (submitted to the Journal of Nutrition).

Author's contribution: AME Hoogeveen was responsible for the *in vivo* study (with technical assistance), the *in vitro* study (with technical assistance) and sample analysis (with technical assistance). She also performed the data analysis, bioinformatics (with assistance from a bioinformatician), statistical analysis (under the guidance of Carlos Montoya) and wrote the draft manuscript.

Abstract

Objective. This study aimed to investigate the contribution of both microbial community and fibre source to *in vitro* ileal fermentation outcomes.

Methods. Six ileal cannulated female pigs (Landrace/Large White; nine-week-old; 30.5 kg bodyweight) were fed diets containing black beans, wheat bread, chickpeas, peanuts, pigeon peas, sorghum, or wheat bran as the sole protein source for seven days (100 g

protein/kg DM diet). On day seven, ileal digesta were collected and stored at -80°C for microbial analysis and *in vitro* fermentation. For each diet, a pooled ileal inoculum was prepared to ferment different fibres (cellulose, pectin, arabinogalactan, inulin, fructooligosaccharides, resistant starch) for 2 hours at 37°C. The OM fermentability and organic acid production were analysed after *in vitro* fermentation. A two-way ANOVA (inoculum x fibre) was used to test the data.

Results. Forty-five percent of the genera identified differed across diets. For instance, the number of *Lactococcus* was 115-fold greater ($P \leq 0.05$) in the ileal digesta of pigs fed the pigeon pea diet than in pigs fed the wheat bran diet. For both *in vitro* organic matter fermentability and organic acid production, there were significant ($P \leq 0.05$) interactions between inoculum and fibre source. For instance, pectin and resistant starch resulted in 1.6- to 31-fold more ($P \leq 0.05$) lactic acid production when fermented by the pigeon pea inoculum than other inocula. For specific fibre sources, statistically significant correlations were found between members of the ileal microbial community and fermentation outcomes.

Conclusion. The fibre source played a greater role in the *in vitro* fermentation than the ileal microbial community of pigs. This role is, however, explained by the substrate preferences of bacterial taxa.

7.1. Introduction

Studies using a combined *in vivo*/*in vitro* fermentation assay in the growing pig have shown that fermentation in the lower small intestine, the ileum, is quantitatively similar to that in the hindgut [286, 287]. For example, the organic matter (OM) fermentability in pigs fed a human-type diet was similar in the ileum (28%) and the hindgut (35%), despite differences in microbial composition and fermentation time [286]. Organic acids, such as acetic and lactic

acids, are found in the ileum of growing pigs [348] and adult humans [154]. These organic acids can be absorbed directly in the ileum [286, 345] and have been found in the bloodstream of human ileostomates [203]. Therefore, ileal fermentation is quantitatively significant and could have important implications for nutrition and health.

The foods (cereals, pulses, etc.) consumed in human diets are diverse in types and amounts of fibre (such as β-glucan in cereals or pectin in fruits). It is known that the type and amount of dietary fibre can change the ileal microbial community in growing pigs [287, 289, 366] and human adults [43, 164], and *in vitro* ileal fermentation outcomes using a porcine inoculum [267, 287]. For example, the partial replacement of dietary cellulose with kiwifruit fibre resulted in a higher α-diversity of the ileal microbial community of growing pigs but a 38% decrease in acetic acid production [287]. However, it is unclear whether the ileal fermentation outcomes were due to changes in the microbial community ('inoculum') or the undigested material entering the ileum ('substrate').

It has been suggested that short-chain fatty acid (SCFA) production during *in vitro* human faecal fermentation is primarily influenced by available substrate rather than inter-individual differences in the microbial community [58]. In contrast, it has been shown that *Prevotella*- and *Bacteroides*-dominated human faecal microbial communities have different *in vitro* fermentative capacities, resulting in different SCFA productions for the fermentation of individual substrates [60]. Moreover, differences across substrates have been reported for *in vitro* fermentation using the same human faecal inoculum [60]. These studies show that hindgut fermentation is influenced by the microbial community and the substrate. Still, the substrate appears to be the major contributor to human faecal fermentation [58, 60]. The ileal microbial community of growing pigs [286, 287] and human adults [12, 20] differs from the hindgut microbial community. It is highly adapted to metabolize simple sugars (like mono-, di- and oligo-saccharides) [12], but it can also utilize polysaccharides [21, 152, 267].

Although it is now well established that there is an active ileal microbiota that ferments undigested material from the upper gastrointestinal tract (GIT), little is known about its fermentative capacity. This study aimed to investigate the effect of the ileal microbial community and fibre source on *in vitro* ileal fermentation and correlated fermentation outcomes with ileal microbial composition using the growing pig as a model for the adult human. It was hypothesised that diets with varying nutrient content would drive changes in the ileal microbial community. These differences in the ileal microbial community and the fibre source fermented were hypothesised to affect *in vitro* ileal fermentation outcomes.

7.2. Materials and methods

7.2.1. Diets

This study was part of a larger study to determine ileal amino acid digestibility in foods commonly consumed by humans [367]. The diets were balanced only for crude protein content, resulting in diets with a wide range of nutrient amounts (Supplementary Table 7.1). For example, the total dietary fibre content ranged from 57 to 295 g/kg DM diet. Previous studies have shown that dietary fibre modulates the ileal microbial community [287]. Thus, this study was a unique opportunity to collect terminal ileal digesta with different microbial communities to determine their contribution to *in vitro* ileal fermentation while applying the reduction principle of the 3Rs associated with animal experimentation.

Only seven out of ten diets used in the larger study were selected for the current study. These seven diets contained whole foods with varying nutrient content (black beans (*Phaseolus vulgaris*), bread (wheat, white), chickpeas (*Cicer arietinum*), peanuts (*Arachis hypogaea*; roasted), pigeon peas (*Cajanus cajan*), sorghum (*Sorghum bicolor*; flour), wheat bran (Kellogg's® All-Bran®)), while the other diets contained a purified protein source and

thus were similar in nutrient content. Each experimental diet was formulated to include one of the test foods as the sole protein source at a concentration of 100 g crude protein/kg DM diet and to meet the growing pigs' nutrient requirements [330]. The test foods were either bought ready to eat or prepared as detailed in the Supplemental methods. Before feeding, each test food was combined with a mixture of non-protein-containing ingredients specific to each diet, containing maize starch, sucrose, vitamin-mineral premix, refined vegetable oil, and cellulose (Supplementary Table 7.1).

7.2.2. In vivo *assay*

Animals and experimental design. The ethical approval for this study was obtained from the Massey University Animal Ethics Committee (Protocol 16/121). The *in vivo* study was conducted according to the previously published protocol [368], with minor adaptations described below. Thirteen female pigs (Landrace/Large White; nine-week-old; 30.5 ± 1.12 kg bodyweight (BW), mean ± SD;) were individually housed in metabolic pens (1.5 x 1.5 m) in a room maintained at 21 ± 2°C with a 12 h/12 h light/dark cycle. Each pig had a T-cannula surgically implanted at the terminal ileum (15 cm before the ileal caecal valve junction). After the surgery, pigs had an eight- to ten-day recovery period, during which they received a basal diet. The pigs had free access to water for the whole duration of the study.

The parent study consisted of nine experimental periods of seven days each. The diets were randomly allocated during experimental periods one to three and six to nine using an incomplete Latin Square design (n = 6 pigs/diet). The pigs received a protein-free and basal diet in periods four and five, respectively. Pigs were given each diet for seven days with their daily ration (80 g DM/kg metabolic BW; $BW^{0.75}$) divided into two equal-sized meals at 0800 and 1700 h. On day seven, ileal digesta were collected three hours post-feeding (2000 h).

A plastic bag flushed with CO_2 was attached to the ileal cannula using a rubber band until approximately 50 g of digesta were collected (between 15 min and 2 h). The collected digesta were mixed, and two aliquots were taken for microbial analysis. The aliquots and the remaining ileal digesta in the plastic bag were stored at -80°C. Supplementary Table 7.2 describes the ileal samples that were collected. Pigs were weighed weekly, and the daily ration was adjusted accordingly.

7.2.3. In vitro *ileal fermentation assay*

For *in vitro* ileal fermentation, a methodology was used that has been optimised for several parameters such as pH, fermentation time, and inoculum concentration [13]. The ileal digesta collected from individual pigs was insufficient to perform the *in vitro* fermentation for each pig, so a pooled inoculum was prepared for each diet by thawing and mixing ileal digesta of all pigs fed the same diet (i.e., an equal amount of wet weight digesta per pig). The inoculum was prepared by combining the pooled digesta (220 g) with one litre of sterilised 0.1 M phosphate-buffered saline (PBS, anaerobic, 4.1 mM L-cystine, pH 7) at 37°C, homogenizing it for 15 sec and straining the mixture through four sterilised layers of cheesecloth to remove large particles. PBS (5 mL) was added to the fermentation bottles containing no fibre (i.e., blanks) or 100 mg fibre source, after which the bottles were flushed with CO_2. Then, 5 mL of the ileal inoculum was added, flushed with CO_2, capped, and incubated at 37°C for two hours [13]. Ten fermentation bottles were used per fibre source or blank. The fibre sources used for the *in vitro* ileal fermentation were cellulose, pectin (from citrus peel), inulin (from chicory), (+)-arabinogalactan (AG; from larch wood), fructooligosaccharides (FOS; from chicory), and high-amylose corn starch (Hylon VII, Ingredion, Auckland, New Zealand) containing 74% type 2 resistant starch [369]. All fibre

sources, except resistant starch, were obtained from Sigma-Aldrich (Auckland, New Zealand). Each inoculum had its own blanks to account for the potential fermentation of material in the inoculum (refer to calculations in the Supplemental methods).

7.2.4. Chemical analysis

The diets were analysed for DM, ash [300], crude protein (nitrogen x 6.25) using an elemental analyser LECO, total lipids using a Soxhlet extractor and petroleum ether extraction, starch using a Total Starch Assay Kit (AA/AMG, Megazyme), total dietary fibre (including soluble and insoluble fibre) [331], and gross energy using a LECO AC-350 Automatic Calorimeter. After *in vitro* ileal fermentation, half the bottles (i.e., n = 5) were analysed for DM and ash [300] to determine OM fermentability. The OM fermentability in the samples was corrected by the OM present in the blanks (i.e., no fibre source added, refer to calculations in the Supplemental methods).

Organic acid analysis. The remaining bottles (i.e., n = 5) were used to determine the organic acid concentration of acetic, propionic, butyric, valeric, iso-butyric, iso-valeric, formic, lactic, and succinic acids using a gas chromatography methodology [302]. Organic acids were derivatised using N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (Sigma-Aldrich). 2-ethylbutyric acid (5 mM) was used as an internal standard. The samples and standards were analysed on a gas chromatograph (GC-2010, Shimadzu) equipped with an SH-Rtx-1 column (30 m x 0.25 mm x 0.25 µm; Shimadzu) and flame ionization detector, using helium as carrier gas [332]. The organic acid production in the samples was corrected by the organic acids present in the blanks (i.e., no fibre source added, refer to calculation in the Supplemental methods). The concentrations of butyric, valeric, iso-butyric, and iso-valeric acids were negligible in the samples (i.e., below the

detection limit). Thus, the total organic acid production was calculated as the sum of formic, acetic, propionic, lactic, and succinic acid concentrations.

7.2.5. Microbial analysis

A Qiagen PowerSoil kit (Qiagen, Australia) was used to extract the DNA from ileal digesta for each pig. The manufacturer's instructions were followed with minor adjustments described previously [287]. The total number of bacteria (i.e., 16S rRNA gene copies) was determined using a quantitative polymerase chain reaction (qPCR), and Illumina sequencing was performed to determine the taxonomic composition (Supplemental methods).

7.2.6. Statistical analysis

A sample size of six animals was required for the parent study. This sample size was also sufficient to reach a statistically significant difference in ileal microbial composition between diet groups with a power >80% at a two-tail 5% significance level, based on previously reported means and variances [287]. For *in vitro* ileal fermentation, between 2 and 6 replicates, i.e., fermentation bottles, were required to detect a statistical difference with a power >80% at a two-tail 5% significance level based on reported means and variance [13]. However, we did not have enough ileal digesta to perform the *in vitro* fermentation for each individual animal. Thus, in this study, we pooled the ileal digesta across animals within each diet. Five technical replicates were used for each diet and fibre source combination. For the microbial analysis, the number of replicates differs per diet because, for some of the samples, the extracted DNA had a concentration or quality that was too low to obtain accurate 16S rRNA gene sequencing results.

The significant separation for the groupings of the ileal samples after performing the Bray-Curtis similarity principal coordinates analysis (PCoA) was tested in Calypso using the Adonis permutational (999 permutations) and betadisper tests. The remaining statistical analyses were done using the SAS software (version 9.4, SAS Institute Inc.). Only microbial taxa with more than 1% relative abundance in at least one sample were included in the statistical analyses. Thus, for the taxonomic composition (number of gene copies per gram ileal digesta), Shannon diversity, and predicted metabolic activity, the effect of the diet was analysed with a one-way ANOVA model using the Proc Mixed procedure. For the taxonomic composition, Shannon Diversity Index, and predicted metabolic activity data, the effect of diet was included in the model as a fixed effect and the period and diet sequence as random effects, with the pig as the experimental unit. For all the response variables, period and diet sequence did not have ($P > 0.05$) a significant effect and therefore were removed from the model.

Some bacterial taxa were not detected or detected in only a few pigs fed the same diet. Therefore, next to the taxonomic composition analysis, a frequency analysis was also performed using a binary logistic regression using the Proc Glimmix procedure with 0 when the taxon was not present and 1 when a taxon had at least one read. No statistical analysis was performed if the frequency was 1 for all diets.

A two-way ANOVA model was used to determine the effect of the inoculum, fibre source, and their interaction on the OM fermentability and productions of total organic acids, formic, acetic, propionic, and lactic acids using the Proc Mixed procedure. Succinic acid production was observed for only one fibre source (i.e., AG). Thus, a one-way ANOVA model was used to test the effect of the inoculum on succinic acid production.

A combination of the ODS Graphics, Proc Univariate procedure, and the Repeated statement of SAS was used to test the model diagnostics of each response variable. The taxonomic composition data and predicted metabolic activity were $\log_{10}$ transformed to achieve homogenous variance. The Repeated statement in the Proc Mixed model allowed testing for the homogeneity of variance by fitting models with the Restricted Maximum Likelihood method and comparing them using the log-likelihood ratio test. The selected model for all response variables had similar Studentised residuals (i.e., equal variances) across treatments. When the F-value of the model was significant ($P \leq 0.05$), the mean values were compared using the adjusted Tukey-Kramer test. Probability values were considered statistically different when $P \leq 0.05$, and values of $0.05 \leq P \leq 0.10$ were considered a trend.

Pearson correlation analyses were performed using the Proc Corr procedure to evaluate the relationship between fermentation outcomes (i.e., OM fermentability and organic acid production) and the bacterial taxa in the inoculum ($n = 7$ inocula per fibre source). It was assumed that the bacterial taxa in the inoculum were the same as the average of the bacterial taxa found in the ileal digesta used to prepare the inoculum (i.e., ileal digesta from individual pigs receiving the same diet).

7.3. Results

All pigs remained healthy and with a daily live weight gain of 501 ± 25 g/day (mean ± SEM). One pig was excluded from the analysis as it displayed coprophagic behaviour.

7.3.1. In vitro *organic matter fermentability*

On average, the *in vitro* ileal OM fermentability of the blanks for all the inocula was 0%, except for the peanut inoculum (4.2 ± 0.44%).

The *in vitro* ileal OM fermentability of the different added fibre substrates ranged from 3 to 37% across all inocula. In general, pectin was significantly more (27.1 ± 0.39% on average across all inocula; $P \leq 0.05$; Table 7.1) fermented than the other fibre sources, while cellulose was significantly less fermented (0.35 ± 0.20%). There was a marked effect of inoculum (i.e., microbial composition). The peanut inoculum supported a significantly greater *in vitro* ileal OM fermentability (19.1 ± 0.52% on average across all fibre sources; $P \leq 0.05$) than the other inocula, and the sorghum inoculum led to a significantly lower OM fermentability of the substrates tested (10.7 ± 0.39%). When considering all data, an interaction ($P < 0.001$) between the inoculum and fibre source was observed for ileal OM fermentability. For example, on average, FOS was 2.6-fold more ($P \leq 0.05$) fermented by the black bean, bread, and peanut inocula than the chickpea, pigeon pea, sorghum, and wheat bran inocula. On the other hand, the black bean inoculum had a 2.1-fold higher ($P \leq 0.05$) OM fermentability of resistant starch than the pigeon pea inoculum.

7.3.1. In vitro *ileal organic acid production*

Across all inocula, AG supported a significantly greater ($P \leq 0.05$) total organic acid production after *in vitro* ileal fermentation than other fibre sources. At the same time, cellulose had a significantly lower ($P \leq 0.05$) total organic acid production (Table 7.2). Across all fibre sources, a significantly greater ($P \leq 0.05$) total organic acid production was obtained with the pigeon pea inoculum compared to the other inocula. When considering all data for total, formic, acetic, propionic, and lactic acid production, consistent with the OM

fermentability results, there was an interaction between inoculum and fibre source ($P < 0.001$). For example, when fermenting FOS, the peanut inoculum produced 2.5-fold higher total organic acids than the wheat bran inoculum. In comparison, the wheat bran inoculum produced 4.3-fold greater total organic acids when fermenting inulin than the peanut inoculum. Depending on the fibre source, some inocula did not produce propionic acid (Supplementary Table 7.3). For example, the peanut inoculum produced propionic acid when fermenting FOS but not when fermenting inulin. Succinic acid was only produced when AG was fermented.

Table 7.1: *In vitro* organic matter fermentability of dietary fibre substrates fermented using pooled ileal inocula from growing pigs fed diets containing different test foods[1]

Inoculum	Fibre substrate						Mean (range)
	AG	Cellulose	FOS	Inulin	Pectin	High-amylose starch	
	%						
Black bean	16.5 ± 1.11^{bc,†‡}	0.00 ± 0.208^{d,§}	21.8 ± 0.653^{b,†}	19.4 ± 1.35^{bc,†‡}	26.5 ± 0.523^{a,‡}	14.8 ± 1.32^{c,†}	16.4 (0.00 - 28.3)
Bread	17.5 ± 0.770^{b,†‡}	1.06 ± 0.408^{d,‡§}	28.7 ± 3.09^{ab,†}	24.1 ± 1.45^{ab,†}	25.2 ± 1.21^{a,†‡}	9.33 ± 0.913^{c,†‡}	17.7 (0.51 - 37.1)
Chickpea	13.9 ± 1.29^{bc,†‡}	3.13 ± 0.583^{d,†‡}	12.8 ± 0.504^{b,‡}	12.1 ± 1.04^{bc,‡§}	25.3 ± 1.71^{a,†‡}	8.90 ± 0.685^{c,†‡}	12.7 (1.99 - 29.1)
Peanut	22.5 ± 1.80^{ab,†}	6.06 ± 0.375^{d,†}	28.8 ± 2.25^{a,†}	16.4 ± 0.745^{b,‡}	30.1 ± 0.544^{a,†}	10.5 ± 0.757^{c,†‡}	19.1 (5.24 - 35.6)
Pigeon pea	15.9 ± 0.739^{b,†‡}	0.00 ± 1.03^{d,§}	8.93 ± 0.407^{c,§}	12.8 ± 1.20^{bc,‡§}	26.9 ± 0.609^{a,†‡}	6.93 ± 0.506^{c,‡}	11.0 (0.00 - 28.3)
Sorghum	15.3 ± 1.26^{b,†‡}	0.00 ± 0.326^{d,§}	7.44 ± 0.496^{c,§}	6.23 ± 1.00^{c,§}	29.1 ± 1.05^{a,†‡}	6.89 ± 1.23^{c,†‡}	10.7 (0.00 - 31.0)
Wheat bran	13.5 ± 0.786^{b,‡}	0.00 ± 0.316^{c,§}	12.1 ± 1.26^{b,‡§}	8.11 ± 0.901^{b,§}	26.6 ± 1.00^{a,†‡}	10.1 ± 0.633^{b,†‡}	11.6 (0.00 - 29.1)
Mean (range)	16.4 (10.1 - 29.1)	0.35 (0.00 - 7.03)	17.2 (6.30 - 37.1)	14.2 (4.37 - 28.8)	27.1 (19.4 - 31.3)	9.63 (3.88 - 18.3)	
	Inoculum (I)	Substrate (S)	I x S				
P value	<0.001	<0.001	<0.001				

[1] Values are means ± SEM per inoculum, *n* = 5 fermentation bottles. When negative values were observed (due to correction by OM found in the blanks), values were assumed to be zero, meaning that OM was not fermented). A two-way ANOVA model was used to assess the effect of inoculum, fibre source substrate, and their interaction. A repeated statement for each fibre source substrate by inoculum combination was required to have similar studentised residuals as described in the statistical analysis section. Means in a row (i.e., fibre source substrate effect) with different letters differ ($P \leq 0.05$), and means in a column (i.e., inoculum effect) with different symbols differ ($P \leq 0.05$). AG, arabinogalactan; FOS, fructooligosaccharides; OM, organic matter.

Table 7.2: Production of total organic, formic, acetic, and lactic acids during *in vitro* fermentation of dietary fibre substrates using pooled ileal inocula from growing pigs fed diets containing different test foods[1]

Organic acid	Inoculum	Fibre substrate						Mean (range)
		AG	Cellulose	FOS	Inulin	Pectin	High-amylose starch	
		mmol/kg DM substrate						
Total	Black bean	384 ± 10.6^{a,‡}	30.7 ± 0.89^{d,§}	68.8 ± 11.9^{bcd,†‡§}	66.1 ± 4.95^{c,‡}	114 ± 5.25^{b,‡}	127 ± 6.57^{b,†‡§}	132 (29.8 - 402)
	Bread	456 ± 15.8^{a,†‡}	50.4 ± 1.03^{c,‡}	99.3 ± 5.53^{b,†‡}	90.3 ± 4.31^{b,†‡}	37.2 ± 2.18$^{d,‖}$	94.1 ± 11.6^{bcd,‡§}	138 (32.7 - 506)
	Chickpea	502 ± 8.20^{a,†}	54.9 ± 3.91^{c,†‡}	110 ± 4.70^{b,†}	88.8 ± 3.85^{b,†‡}	42.0 ± 13.1$^{cbc,§‖}$	97.0 ± 3.14^{b,§}	149 (18-8 - 518)
	Peanut	480 ± 27.1^{a,†‡}	76.8 ± 2.63^{c,†}	130 ± 6.06^{b,†}	33.1 ± 6.95^{d,§}	96.2 ± 15.9$^{bc,‡§‖}$	124 ± 3.88^{b,‡}	157 (19.2 - 547)
	Pigeon pea	477 ± 24.0^{a,†‡}	75.2 ± 7.38^{c,†‡}	105 ± 10.1^{c,†‡§}	62.3 ± 8.65^{c,†‡§}	197 ± 7.02^{b,†}	174 ± 8.61^{b,†}	182 (47.6 - 535)
	Sorghum	402 ± 11.2^{a,‡}	120 ± 13.4^{b,†}	48.3 ± 10.9^{b,‡c}	116 ± 15.9^{b,†‡§}	77.8 ± 5.61^{b,‡§}	102 ± 13.1^{b,†‡§}	144 (26.9 - 436)
	Wheat bran	525 ± 33.1^{a,†‡}	38.1 ± 8.63^{cd,‡§}	51.7 ± 4.51^{c,§}	141 ± 13.9^{b,†}	18.3 ± 4.41$^{d,‖}$	135 ± 10.9^{b,†‡§}	152 (10.1 - 581)
	Mean (range)	461 (356 - 581)	63.7 (15.7 - 133)	87.5 (26.9 - 141)	85.4 (19.2 - 179)	83.1 (10.1 - 216)	122 (73.1 - 200)	
Formic acid	Black bean	323 ± 17.8^{a}	3.52 ± 2.87^{c,§}	8.68 ± 2.43^{c,‡§}	5.59 ± 4.48^{c,§}	8.17 ± 1.51^{c,‡}	28.3 ± 3.84^{b,‡§}	62.8 (3.00 - 337)
	Bread	324 ± 15.9^{a}	29.2 ± 2.87^{b,†}	16.3 ± 2.43^{b,†‡§}	21.0 ± 3.47^{b,‡§}	2.28 ± 2.14^{c,‡}	27.8 ± 3.84^{b,‡§}	70.1 (2.07 - 351)
	Chickpea	304 ± 15.9^{a}	6.57 ± 2.03^{c,§}	26.8 ± 2.11^{b,†}	6.74 ± 5.49^{bc,§}	3.41 ± 2.14^{c,‡}	28.8 ± 2.72^{b,‡}	62.7 (3.16 - 319)

	Peanut	348 ± 15.9^{a}	7.17 ± 2.03^{c,§}	26.8 ± 2.11^{b,†}	17.0 ± 3.47^{bc,‡§}	9.64 ± 1.65^{c,‡}	31.2 ± 2.43^{b,‡}	73.2 (3.40 - 385)
	Pigeon pea	323 ± 15.9^{a}	21.4 ± 2.03^{b,†‡}	7.30 ± 2.11^{c,‡§}	7.34 ± 4.48^{bc,§}	10.8 ± 1.51^{bc,‡}	9.21 ± 3.14^{bc,§}	63.1 (4.48 - 343)
	Sorghum	348 ± 15.9^{a}	3.68 ± 4.06^{e,‡§}	26.1 ± 2.98^{d,†‡}	56.4 ± 3.88^{bc,†}	41.3 ± 1.74^{c,†}	76.3 ± 2.72^{b,†}	92.0 (3.68 - 398)
	Wheat bran	375 ± 15.9^{a}	10.9 ± 2.03^{c,‡§}	11.0 ± 2.43^{c,‡§}	37.6 ± 3.47^{b,†‡}	11.4 ± 1.74^{c,‡}	43.1 ± 2.43^{b,‡}	81.6 (7.01 - 447)
	Mean (range)	335 (284 - 447)	11.8 (3.00 - 34.7)	17.6 (4.48 - 33.3)	21.7 (5.02 - 80.7)	12.4 (2.07 - 46.3)	35.0 (7.11 - 80.1)	
Acetic acid	Black bean	58.1 ± 1.48^{a,§}	4.23 ± 2.10^{c,§}	23.8 ± 1.71^{b,‡§}	8.5 ± 1.71^{c,§}	11.6 ± 1.71^{c,‡§}	14.6 ± 1.48^{bc,‡}	20.1 (3.89 - 64.4)
	Bread	44.0 ± 1.96$^{a,\|\|}$	7.26 ± 3.11^{b,§}	40.8 ± 3.11^{a,†}	19.2 ± 2.54^{b,‡§}	21.0 ± 1.96^{b,‡}	20.4 ± 2.54^{b,†‡}	25.4 (7.09 - 47.7)
	Chickpea	70.8 ± 2.09^{a,†‡}	12.8 ± 2.70^{d,‡§}	52.3 ± 2.34^{b,†}	43.0 ± 2.70^{bc,†}	2.01 ± 3.31^{d,§}	33.3 ± 2.34^{c,†}	35.7 (1.98 - 77.9)
	Peanut	72.6 ± 4.57^{a,†‡§}	26.5 ± 5.89^{b,‡§}	14.7 ± 5.10^{b,‡}	14.4 ± 5.89^{b,‡§}	43.3 ± 4.57^{b,†}	23.0 ± 5.10^{b,†‡}	32.4 (10.8 - 85.1)
	Pigeon pea	88.9 ± 3.76^{a,†}	30.8 ± 3.76^{b,‡}	39.9 ± 4.20^{b,†‡§}	2.75 ± 4.20^{c,§}	30.0 ± 4.20^{b,†‡}	17.4 ± 4.20^{bc,†‡}	35.0 (1.55 - 97.6)
	Sorghum	17.1 ± 10.14$^{b,\|\|}$	123 ± 10.1^{a,†}	25.6 ± 10.1^{b,†‡§}	47.3 ± 7.85^{b,†‡§}	19.9 ± 12.4^{b,†‡§}	37.8 ± 14.2^{b,†‡}	45.2 (11.2 - 152)
	Wheat bran	60.5 ± 3.56$^{a,\ddagger\S\|\|}$	20.2 ± 4.11^{b,‡§}	7.39 ± 5.03^{b,§}	19.9 ± 4.11^{b,‡§}	12.1 ± 7.12^{b,†‡§}	29.3 ± 3.18^{b,†‡}	24.9 (4.09 - 74.9)
	Mean (range)	58.9 ± 1.81 (14.7 - 97.6)	32.1 ± 1.97 (3.89 - 152)	29.2 ± 1.97 (4.09 - 58.8)	22.1 ± 1.74 (1.55 - 70.0)	20.0 ± 2.31 (1.98 - 61.8)	25.1 ± 2.13 (10.9 - 45.4)	
Lactic acid	Black bean	11.0 ± 6.66$^{c,\|\|}$	22.6 ± 6.66^{bc,†‡§}	32.4 ± 4.71^{bc,†‡}	49.1 ± 4.71^{b,†‡}	91.6 ± 6.66^{a,‡}	95.2 ± 4.21^{a,‡}	50.3 (9.07 - 113)
	Bread	80.4 ± 4.50^{a,‡}	6.17 ± 5.03^{d,§}	40.2 ± 4.50^{bc,†}	47.4 ± 4.50^{b,†‡}	14.0 ± 4.50$^{cd,\|\|\#}$	36.6 ± 4.50$^{bc,\|\|}$	37.5 (4.19 - 96.5)

	Chickpea	119 ± 3.51^{a,†}	21.8 ± 4.96^{c,†‡§}	26.5 ± 3.51^{c,†‡}	49.9 ± 4.05^{d,†}	63.3 ± 7.02^{b,‡§}	31.8 ± 3.14$^{bc,\|\|}$	52.0 (19.2 - 122)
	Peanut	52.2 ± 4.68^{a,§}	42.0 ± 4.68^{ab,†}	7.14 ± 6.05^{c,‡§}	15.8 ± 7.41^{bc,‡§}	45.1 ± 6.05$^{ab,\S\|\|}$	11.3 ± 5.24$^{c,\|\|\#}$	28.9 (3.56 - 68.9)
	Pigeon pea	80.1 ± 9.33^{b,†‡§}	43.2 ± 10.8^{b,†‡§}	42.6 ± 10.7^{b,†‡§}	54.7 ± 9.33^{b,†‡}	155 ± 9.33^{a,†}	152 ± 8.34^{a,†}	88.1 (38.4 - 181)
	Sorghum	53.8 ± 1.63^{a,§}	22.8 ± 1.89^{b,†‡}	4.42 ± 1.63^{d,§}	6.07 ± 1.63^{d,§}	17.5 ± 1.63$^{bc,\#}$	9.11 ± 1.89$^{cd,\#}$	18.9 (2.08 - 58.3)
	Wheat bran	58.7 ± 2.93^{a,‡§}	15.1 ± 3.68^{c,‡§}	38.2 ± 2.93^{b,†}	38.3 ± 3.38^{b,†‡}	4.99 ± 2.93$^{c,\#}$	65.6 ± 2.93^{a,§}	368 (1.54 - 73.2)
	Mean (range)	65.0 (9.07 - 122)	24.8 (4.19 - 48.2)	27.4 (2.08 - 50.8)	37.3 (5.14 - 76.1)	56.0 (1.54 - 181)	57.4 (7.56 - 175)	

P value		Inoculum (I)	Substrate (S)	I x S
	Total	<0.001	<0.001	<0.001
	Formic acid	<0.001	<0.001	<0.001
	Acetic acid	<0.001	<0.001	<0.001
	Lactic acid	<0.001	<0.001	<0.001

[1] Values are means ± SEM, n = 5 fermentation bottles. A two-way ANOVA model was used to assess the effect of inoculum, fibre source substrate, and their interaction for total organic, formic, acetic, and lactic acids. A different repeated statement was required for each organic acid to have similar studentised residuals as described in the statistical analysis section. For example, the best-repeated statement for formic acid had a common SEM per fibre source substrate, while for acetic acid, it had a common SEM per inoculum. Means in a row (i.e., inoculum effect) with different letters differ ($P \leq 0.05$), and means in a column (i.e., fibre source substrate effect) with different symbols differ ($P \leq 0.05$). The butyric, valeric, iso-butyric, and iso-valeric acid productions were negligible in the samples (i.e., below the detection limit) and, therefore, not reported. The means of propionic and succinic acid productions are reported in Supplementary Table 3. AG, arabinogalactan; DM, dry matter; FOS, fructooligosaccharides; ND, not detected.

7.3.2. Ileal microbial composition

In the current study, seven phyla and 57 genera were identified (>1% relative abundance in at least one sample). Six phyla (86%) and twenty-seven genera (47%) were present in ileal digesta for all diets at a 100% frequency. The remaining taxa displayed different frequencies across diets (Supplementary Table 7.4). For example, *Leuconostoc* had a higher (0.83 versus < 0.25; $P \leq 0.05$) frequency in the ileal digesta of pigs fed the sorghum diet than in those given the bread, chickpea, peanut, or wheat bran diets.

The pigs fed the bread diet had a 3.5-fold higher total number of bacteria in their ileal digesta than pigs fed the sorghum and wheat bran diets (based on back-transformed data; Table 7.3). Forty-five percent of the total genera identified differed ($P \leq 0.05$) across diets (Supplementary Table 7.5). For example, the number of Proteobacteria was on average 3.3-fold higher ($P \leq 0.05$) in the ileal digesta of pigs fed the bread and sorghum diets than those given the wheat bran diet (based on back-transformed data). Based on PICRUSt, the predicted metabolic activity of the ileal microbial composition of pigs was similar across all diets, with a few exceptions (Supplementary Table 7.6).

The α-diversity (Shannon Diversity Index) of the ileal microbial community was similar ($P = 0.665$) across all diets (Supplementary Figure 7.1). The Bray-Curtis dissimilarity index (i.e., β-diversity) did not clearly separate the diets for the ileal microbial community pertaining to individual pigs (Figure 7.1). However, an Adonis test suggested that the diets were significantly different ($P < 0.001$). A betadine test for intragroup variance was significant ($P = 0.036$), indicating that some differences between ileal microbial compositions across diets could be ascribed to greater intragroup variability.

Table 7.3: Effect of diet on numbers of bacteria in ileal digesta from growing pigs (across substrates) fed diets containing different test foods[1]

Phylum	Genera	Diet							SEM	*P* value
		Black bean	Bread	Chickpea	Peanut	Pigeon pea	Sorghum	Wheat bran		
Sample size, *n*[2]		4	5	4	5	6	6	6		
		Log_{10} 16S rRNA gene copies/g wet digesta								
Total bacteria		10.6^{ab}	11.1^{a}	10.8^{ab}	10.9^{ab}	11.1^{ab}	10.6^{b}	10.3^{b}	0.180	0.002
Actinobacteria										
	Actinomyces	7.67^{ab}	8.32^{ab}	7.95^{ab}	7.82^{ab}	8.30^{a}	7.66^{ab}	7.43^{b}	0.192	0.036
	Bifidobacterium	7.73^{a}	6.77^{ab}	8.29^{ab}	7.34^{ab}	7.71^{ab}	5.98^{b}	6.87^{ab}	0.556	0.009
	Collinsella	6.99^{ab}	7.61^{a}	6.68^{ab}	7.74^{ab}	7.11^{ab}	6.04^{b}	7.47^{a}	0.357	0.010
Bacteroidetes		9.79^{b}	10.3^{a}	9.87^{ab}	10.3^{ab}	10.2^{ab}	9.59^{ab}	9.83^{ab}	0.164	0.020
	Bacteroides	9.29^{ab}	9.79^{a}	9.33^{ab}	9.81^{ab}	9.67^{ab}	9.17^{ab}	8.64^{b}	0.229	0.038
	Parabacteroides	7.47^{b}	8.23^{a}	7.50^{abc}	7.79^{abc}	7.92^{abc}	7.89^{ab}	6.73^{c}	0.252	0.001
	Prevotella	9.29^{ab}	9.81^{a}	9.29^{ab}	9.45^{ab}	9.89^{ab}	8.93^{b}	9.71^{a}	0.205	0.009
Firmicutes		10.3^{ab}	10.9^{a}	10.6^{ab}	10.5^{ab}	10.8^{ab}	10.2^{b}	9.85^{b}	0.200	0.005
	Agathobacter	7.15^{ab}	7.52^{ab}	6.93^{ab}	7.28^{ab}	6.54^{ab}	6.14^{b}	7.86^{a}	0.352	0.027
	Anaerovibrio	6.66^{bc}	7.40^{b}	7.39^{ab}	8.09^{abc}	7.10^{abc}	5.74^{c}	8.82^{a}	0.398	<0.001
	Blautia	7.25^{b}	7.44^{ab}	7.17^{ab}	7.81^{ab}	7.45^{ab}	7.18^{b}	8.47^{a}	0.329	0.008
	Clostridium_sensu_stricto_1	8.54^{ab}	10.1^{a}	9.53^{ab}	9.62^{ab}	9.53^{ab}	8.98^{b}	8.13^{c}	0.309	0.035
	Enterococcus	8.63^{a}	8.13^{a}	6.85^{b}	7.65^{ab}	8.47^{ab}	7.62^{a}	7.96^{ab}	0.379	0.007
	Faecalibacterium	6.54^{bc}	6.47^{bc}	6.57^{bc}	6.57^{bc}	6.61^{b}	5.69^{c}	8.44^{a}	0.315	<0.001
	Fusicatenibacter	6.08^{b}	6.14^{b}	5.84^{b}	ND	6.12^{b}	5.81^{b}	7.71^{a}	0.241	0.027
	Lachnospiraceae_unclassified	7.98^{b}	8.63^{a}	8.22^{ab}	8.71^{ab}	8.56^{ab}	8.30^{ab}	8.32^{ab}	0.192	0.019

Lactococcus	7.37^{ab}	6.66^{ab}	6.84^{ab}	6.19^{ab}	7.85^{a}	7.97^{a}	5.79^{b}	0.432	0.007
Megamonas	7.62^{ab}	7.77^{abc}	7.95^{ab}	6.79^{bc}	7.63^{abc}	5.96^{c}	8.79^{a}	0.371	<0.001
Megasphaera	7.02^{ab}	7.14^{a}	8.19^{ab}	7.32^{ab}	7.67^{a}	5.67^{b}	6.82^{a}	0.486	0.001
Romboutsia	8.61^{abc}	9.81^{a}	9.26^{ab}	9.24^{ab}	9.41^{ab}	9.07^{b}	7.24^{c}	0.236	0.001
Sarcina	9.09^{ab}	10.3^{a}	8.37^{ab}	8.18^{ab}	9.91^{a}	8.30^{ab}	6.54^{b}	0.554	0.003
Selenomonadaceae_unclassified	7.33^{abc}	9.35^{abc}	9.66^{a}	7.37^{c}	9.43^{ab}	7.42^{abc}	7.70^{bc}	0.376	0.019
Streptococcus	9.45^{ab}	9.80^{a}	9.31^{ab}	9.22^{ab}	9.49^{ab}	9.23^{ab}	8.75^{b}	0.290	0.039
Terrisporobacter	8.60^{ab}	9.62^{a}	9.26^{a}	8.91^{a}	8.63^{ab}	8.87^{a}	7.16^{b}	0.330	<0.001
Turicibacter	9.13^{ab}	9.52^{a}	9.54^{a}	9.67^{a}	9.45^{a}	8.68^{a}	7.58^{b}	0.324	<0.001
Veillonella	8.65^{ab}	9.19^{ab}	9.28^{a}	8.77^{ab}	9.36^{ab}	8.11^{b}	8.78^{ab}	0.297	0.030
Fusobacteria	9.43^{b}	10.0^{a}	9.37^{b}	10.0^{ab}	9.79^{ab}	9.36^{b}	9.16^{b}	0.166	0.002
Fusobacterium	9.42^{b}	10.0^{a}	9.36^{b}	10.0^{ab}	9.76^{ab}	9.35^{b}	9.16^{b}	0.164	0.002
Proteobacteria	10.0^{ab}	10.1^{a}	9.59^{ab}	10.1^{ab}	10.2^{ab}	10.0^{a}	9.56^{b}	0.180	<0.001
Acinetobacter	7.28^{ab}	7.32^{ab}	6.24^{b}	6.77^{ab}	6.87^{ab}	8.31^{a}	6.73^{ab}	0.293	0.009
Enterobacterales_unclassified	7.58^{ab}	7.82^{a}	7.32^{ab}	7.80^{ab}	7.84^{ab}	7.91^{ab}	7.12^{b}	0.223	0.013
Kosakonia	6.99^{ab}	6.46^{ab}	ND	6.06^{ab}	7.36^{ab}	7.43^{a}	5.36^{b}	0.458	0.009
Pseudomonas	5.77^{bc}	6.26^{b}	ND	6.22^{bc}	6.49^{ab}	8.50^{a}	5.37^{c}	0.269	0.001
Sutterella	7.86^{ab}	8.43^{ab}	8.11^{ab}	8.67^{a}	8.31^{ab}	7.19^{b}	8.17^{ab}	0.326	0.035

[1] Values are means with pooled SEM, *n* = 4-6 animals per diet. Only taxa with >1% relative abundance in at least one of the samples and that showed a significant diet treatment effect (Supplementary Table 5) are presented. The number of 16S rRNA gene copies per taxa was obtained by multiplying the total number of 16S rRNA gene copies with the relative abundance of the taxa with the assumption that each taxon has an equal number of 16S rRNA gene copies. Data were $\log_{10}$ transformed to achieve homogenous variance. A one-way ANOVA model was used to assess the effect of diet. Means in a row with a different letter differ ($P < 0.05$). ND, not detected.

[2] *n* indicates the number of replicates. The different numbers of replicates resulted from either removing one pig that displayed coprophagy or the extracted DNA having low quality or concentration for 16S rRNA gene sequencing.

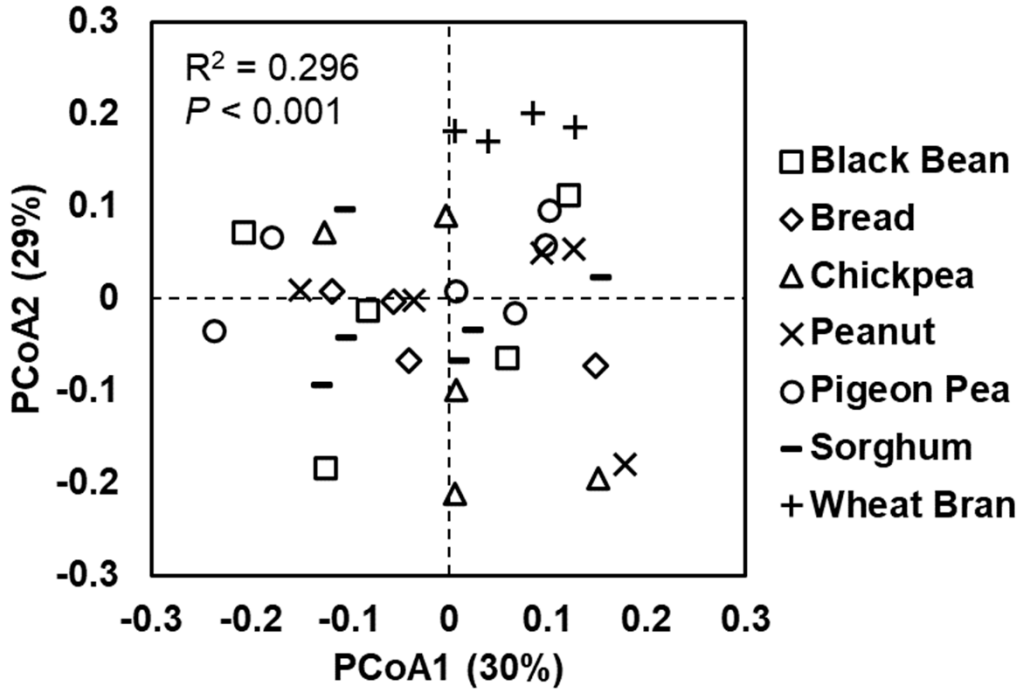

Figure 7.1: Principal coordinate analysis (PCoA) plot of Bray-Curtis dissimilarity of the ileal microbial community for growing pigs fed diets for seven days containing different test foods. Individual symbols represent individual samples, *n* = 4-6 animals per diet. The effect of diet on the groupings was assessed using the Adonis test (999 permutations).

7.3.3. Correlations between the ileal microbial composition and fermentation outcomes

Given that there were marked differences in the number of specific bacteria in the ileal digesta of the pigs, it was of interest to investigate whether the observed differences in the fermentation outcomes were correlated with differences in the bacterial taxa found in the inocula. Correlation coefficients were determined across inocula for each fibre source between OM fermentability or organic acid production and bacterial taxa. Fifteen statistically significant correlations ($P \leq 0.05$; fourteen positive and one negative) were found between OM fermentability and the concentration of bacterial taxa in the ileal digesta across the fibre

sources. For example, *Fusobacterium* was positively correlated with the OM fermentability of AG (r = 0.805; P = 0.029) (Table 7.4). In addition, 39 positive and 35 negative correlations ($P \leq 0.05$) were found between organic acid production and the bacterial taxa for specific fibre sources. For example, *Bifidobacterium* was positively correlated with formic acid when fermenting AG (r = 0.816; P = 0.025) but negatively correlated when fermenting inulin (r = -0.916; P = 0.004) and starch (r = -0.775; P = 0.041).

7.4. Discussion

In the present work, different diets were fed to growing pigs, ileal digesta were collected, and ileal inocula were prepared for *in vitro* fermentation of a wide range of fibre sources. The study showed that the ileal microbial composition, obtained by pooling ileal digesta from pigs fed diets containing different test foods for seven days, influenced the *in vitro* fermentation of fibre sources. As hypothesised, both the inoculum and fibre source influenced the *in vitro* ileal fermentation resulting in differences in OM fermentability and organic acid production. However, the fibre source had a greater effect on these fermentation outcomes than the inoculum. Due to the limited amount of digesta collected per animal, a pooled inoculum was prepared per diet. Thus, the current results do not reflect the variability across animals. Still, they do characterize the fermentability of different fibre sources across different microbial compositions.

Table 7.4: Pearson's correlation coefficients between fermentation outcomes (OM fermentability and organic acid production) and the number of bacteria in the ileal inoculum per fibre substrate after *in vitro* fermentation[1]

Fibre substrate	Bacteria, log_{10} 16S rRNA gene copies/g wet digesta	OM fermentability, %	Organic acid production, mmol/kg DM substrate				
			Total	Formic acid	Acetic acid	Propionic acid	Lactic acid
AG	Actinobacteria	-	-0.77 (0.044)	-	-	-	-
	Bifidobacterium	-	-	0.82 (0.025)	-	-	-
	Bacteroidetes						
	Alloprevotella	-	-	-	-	0.75 (0.040)	-
	Muribaculaceae_unclassified	0.81 (0.029)	-	-	-	-	-
	Firmicutes	-	-0.76 (0.049)	-	-	-	-
	Anaerovibrio	-	0.83 (0.020)	-	-	-	-
	Blautia	-	-	0.81 (0.028)	-	-	-
	Lactobacillus	-	-	-	-	-	0.79 (0.033)
	Megasphaera	-	-	-	0.85 (0.017)	-	-
	Selenomonadaceae_unclassified	-	-	-	-	-	0.85 (0.016)
	Turicibacter	-	-	-0.79 (0.036)	-	-	-
	Fusobacteria	0.80 (0.032)	-	-	-	-	-
	Fusobacterium	0.81 (0.029)	-	-	-	-	-
	Proteobacteria	0.80 (0.031)	-	-	-	-	-
	Acinetobacter	-	-0.77 (0.043)	-	-0.84 (0.018)	-	-
	Enterobacteriaceae_unclassified	-	-0.82 (0.024)	-	-	-	-
	Escherichia_Shigella	0.82 (0.023)	-	-	-	-	-
	Spirochaetes	0.78 (0.039)	-	-	-	0.82 (0.023)	-
	Treponema	0.78 (0.038)	-	-	-	0.82 (0.026)	-

Cellulose	Actinobacteria	-	-	-	-	0.79 (0.034)	-
	Actinomyces	-	-	0.79 (0.036)	-	-	-
	Bacteroidetes						
	Alloprevotella	-	-	-	-	-0.85 (0.016)	-
	Prevotella	-	-	0.82 (0.023)	-	-	-
	Prevotellaceae_NK3B31_group	-	-	0.76 (0.048)	-0.72 (0.068)	-	-
	Firmicutes						
	Agathobacter	-	-0.79 (0.035)	-	-0.73 (0.063)	-	-
	Lachnoanaerobaculum	-	-	0.97 (<0.001)	-	-	-
	Megamonas	-	-0.85 (0.015)	-	-0.77 (0.044)	-	-
	Megasphaera	-	-	-	-0.76 (0.045)	0.78 (0.040)	-
	Proteobacteria						
	Acinetobacter	-	-	-	0.76 (0.049)	-	-
	Spirochaetes	0.78 (0.039)	-	-	-	0.82 (0.023)	-
	Tenericutes						
	Mycoplasma	-	-	0.79 (0.035)	-	-	-
FOS	Total bacteria	-	0.77 (0.043)	-	-	-	-
	Actinobacteria	-	0.93 (0.002)	-	0.71 (0.074)	-	-
	Bacteroidetes	-	0.81 (0.028)	-	-	-	-
	Alloprevotella	-	-	-	-	0.84 (0.017)	-
	Bacteroides	-	0.81 (0.028)	-	-	-	-
	Prevotella	-	-	-	-	-	0.79 (0.036)
	Prevotellaceae_unclassified	-	0.76 (0.048)	-	-	-	-
	Firmicutes	-	0.74 (0.056)	-	0.76 (0.045)	-	-
	Clostridium_sensu_stricto_1	-	0.78 (0.039)	-	-	-	-
	Enterococcus	-	-	-0.86 (0.014)	0.83 (0.021)	-	-

	Lachnoanaerobaculum	-	-	-	-	0.77 (0.043)	-
	Megamonas	-	-	-	-	0.82 (0.024)	-
	Megasphaera	-	0.76 (0.049)	-	-	-	-
	Selenomonadaceae_unclassified	-	-	-	0.87 (0.011)	-	-
	Turicibacter	-	0.83 (0.022)	-	-	-	-
	Proteobacteria	-					
	Sutterella	-	0.77 (0.041)	-	-	-	-
	Spirochaetes	-	-	-	-	0.87 (0.011)	-
	Treponema	-	-	-	-	0.86 (0.013)	-
Inulin	Actinobacteria						
	Bifidobacterium	-	-	-0.92 (0.004)	-	-	-
	Bacteroidetes						
	Bacteroides	-	-0.82 (0.024)	-	-	-	-
	Muribaculaceae_unclassified	-	-0.83 (0.021)	-	-0.81 (0.028)	-	-
	Prevotellaceae_unclassified	0.79 (0.033)	-0.73 (0.063)	-	-	-	-
	Firmicutes						
	Enterococcus	-	-	-	-0.80 (0.029)	-	-
	Megasphaera	-	-	-0.87 (0.010)	-	-	0.71 (0.076)
	Streptococcus	0.76 (0.048)	-	-	-	-	-
	Turicibacter	-	-0.83 (0.021)	-	-	-	-
	Veillonella	-	-	-0.74 (0.056)	-	-	0.81 (0.028)
	Proteobacteria						
	Escherichia_Shigella	0.77 (0.044)	-0.86 (0.014)	-	-0.72 (0.069)	-	-
	Pasteurellaceae_unclassified	0.77 (0.045)	-	-	-	-	-
	Tenericutes						
	Mycoplasma	0.86 (0.013)	-	-	-	-	-

Pectin	Bacteroidetes						
	Muribaculaceae_unclassified	-	-	-	0.76 (0.048)	-	-
	Prevotellaceae_NK3B31_group	-	-	-0.80 (0.032)	-	-	-
	Prevotellaceae_UCG003	0.82 (0.022)	-	-	0.73 (0.061)	0.73 (0.061)	-
	Firmicutes						
	Lactobacillus	-	-	-	-	-0.80 (0.032)	-
	Megasphaera	-	-	-0.84 (0.017)	-	-	-
	Selenomonadaceae_unclassified	-	-	-	-	-0.83 (0.020)	-
	Veillonella	-	-	-0.82 (0.025)	-	-	-
	Proteobacteria	-	-	-	0.86 (0.014)	-	-
	Acinetobacter	-	-	0.80 (0.032)	-	-	-
	Sutterella	-	-	-0.81 (0.027)	-	-	-
High-amylose starch	Actinobacteria						
	Bifidobacterium	-	-	-0.78 (0.041)	-	-	-
	Bacteroidetes						
	Muribaculaceae_unclassified	-	-	-	-0.76 (0.047)	-	-
	Firmicutes						
	Enterococcus	-	-	-	-0.79 (0.033)	-	-
	Lactobacillus	-0.76 (0.047)	-	-	-	-	-
	Megasphaera	-	-	-0.85 (0.015)	-	-	-
	Veillonella	-	-	-0.87 (0.010)	-	-	-
	Fusobacteria						
	Leptotrichia	-	-	-0.80 (0.031)	-	-	-
	Proteobacteria						
	Escherichia_Shigella	-	-	-	-0.80 (0.032)	-	-

Sutterella	-	-	-0.76 (0.046)	-	-	-
Tenericutes						
Mycoplasma	-	-	-	-0.80 (0.029)	-	-

[1] Values are correlation coefficients with *P* values between parentheses. Only taxa with >1% relative abundance in at least one sample were considered. Bacteria data were $\log_{10}$ transformed to achieve homogenous variance. Only significant ($P \leq 0.05$) correlations with a correlation coefficient above 0.7 were included in this table. AG, arabinogalactan; DM, dry matter; FOS, fructooligosaccharides; OM, organic matter.

7.4.1. In vitro *organic matter fermentability*

Firstly, feeding growing pigs with diets of varying nutrient contents (for example, 57 to 295 g total dietary fibre/kg diet) for seven days resulted in different ileal microbial compositions. Dietary interventions have previously been shown to be a successful tool for changing the ileal microbial community in growing pigs with longer interventions, namely 22 [289], 28 [366], and 42 days [287]. However, in humans, the ileal microbial community was already affected after a two-week intervention [164] or even after a single meal [43, 163]. The different ileal microbial communities obtained through dietary intervention allowed us to interrogate our other hypothesis regarding the effect of the ileal microbial community and fibre source on *in vitro* ileal fermentation.

The ileal OM fermentation was quantitatively significant (3 to 37%), as previously found for the *in vitro* fermentation of jejunal digesta [286, 287] and fibre sources [13, 267]. The differences in ileal OM fermentability among fibre sources could be explained by several factors, such as (i) structural differences between the fibre sources, such as monomeric composition and branching [21, 154, 359]. For example, in the current study, pectin that consists of multiple monomeric units (including galacturonic acid, rhamnose, arabinose, and galactose [370]) had a greater *in vitro* OM fermentability than FOS and inulin that contain only fructose monomers [371]; (ii) composition and diversity of the ileal microbial communities. This is supported by the fifteen correlations found in the current study between bacterial taxa and OM fermentability; (iii) fibre source preference of specific bacteria. For example, in the current study, *Escherichia-Shigella* had (or tended to have) a positive correlation with the OM fermentability of AG, FOS, and inulin.

The statistical results showed that ileal OM fermentability was influenced by the interaction between the fibre source and the inoculum. However, considering the higher variability

between fibre sources (27% was the lowest difference in range) compared to the variability between inocula (7% was the lowest difference in range), it appears that the fibre source plays a greater role in ileal fermentation. Similarly, using an inoculum from human ileostomates, both the fibre source and the donor affected the *in vitro* ileal fermentation, but the contribution of the fibre source appeared to be greater [21] despite methodological differences to this study.

7.4.2. In vitro *organic acid production*

In vitro organic acid production was also investigated here to measure the extent of microbial metabolism. Of the organic acids determined in this study, only formic, acetic, and lactic acids were produced in considerable amounts. In contrast, propionic and succinic acids were produced only for specific fibre sources and inocula. Formic acid was the primary fermentation product for the *in vitro* fermentation of AG. On average, it represented 73% of the total organic acids produced, followed by lactic and acetic acids (14% and 13%, respectively). Similar results were found when fermenting *in vitro* AG with a continuous culture of *Bifidobacterium longum* [316] or in a batch fermentation using an ileal inoculum from pigs fed a human-type diet [267].

Based on the variability between fibre sources and inocula for organic acid production, the fibre sources appear to play a greater role in organic acid production during *in vitro* ileal fermentation than the microbial composition, as described above for ileal OM fermentability. A previous study using human faecal inocula from different individuals has also shown that the substrate plays a greater role than the microbial community during *in vitro* faecal fermentation [58]. An important number of statistically significant correlations were fibre source dependent. Some of the factors described above can explain this, such as structural

differences between fibre sources [359, 372] and fibre source preferences of bacteria [60]. For example, *Bifidobacteria* had (or tended to have) a positive correlation with acetic acid production from AG but tended to be negatively correlated with acetic acid production from cellulose. This observation could be regarded as both involving fibre source preference and structural differences. Previous reports have shown that *Bifidobacterium longum* can utilize AG, resulting in acetic acid production in a monoculture [316], but no reports for cellulose were found.

No butyric acid production was detected. Previously, a small amount of butyric acid (i.e., 0.6 mmol/kg DM substrate) was produced when the same fibre sources were fermented *in vitro* with ileal digesta from pigs fed a human-type diet [267] or human ileostomates [21]. The negligible butyric acid production agrees with low butyrate concentrations detected in the ileal digesta of pigs [373, 374] and human adults [23]. In addition, a reduced number of genes related to butyrate fermentation in human ileal microbiota compared to faecal microbiota [12]. Also, most human GIT microbiota form butyrate via the butyryl coenzyme A-acetyl coenzyme A transferase pathway, which requires acetic acid [42]. The two-hour fermentation time in this study may not have been sufficient to allow cross-feeding to occur.

At first sight, there appears to be a discrepancy between the OM fermentability observations and the organic acid data. For example, AG, FOS, and inulin were all fermented similarly (about 16%), but the total organic acid production for AG was about five times higher than that for FOS and inulin. This may be partly explained by the fact that most of the organic acid production for AG found in the current study was from formic acid, which has a relatively low molecular weight [375]. Except in the case of the peanut inoculum, the fermentation of cellulose was negligible. Yet, the total organic acid production was only slightly lower than that of FOS, inulin, and pectin. There is no obvious explanation for this, and it may indicate that the organic acid correction after fermentation of the blanks in this assay may not be

completely accurate. The inoculum contains undigested material from the ileal digesta providing between 35 to 50% of the total OM in the fermentation bottle. The fermentation of this material could potentially differ with and without (i.e., blanks) the added fibre substrate. For instance, in the current study, only the peanut inoculum was fermented. Differences in total organic acid production versus OM fermentability may also be due to differences in the production of metabolites and gases [22] that were not determined here. These unexplained discrepancies mean that the organic acid data should be interpreted cautiously. Overall, however, they provide corroborating evidence for the effect of dietary composition on *in vitro* ileal fermentation.

7.5. Conclusion

In conclusion, *in vitro* ileal fermentation of different well-characterised fibre sources was quantitatively significant when using the growing pig as an animal model for the adult human. There was a statistically significant effect of dietary composition on fermentation. The fibre source and the inoculum influenced the *in vitro* OM fermentability and the organic acids produced. However, based on the source of variation, the fibre source made a greater contribution to ileal fermentation. However, based on significant correlations, the contribution of the fibre source was influenced by the number of specific bacteria. For example, the number of *Prevotellaceae* UCG003 was only related to the *in vitro* ileal fermentation of pectin. These results suggest that dietary intervention could play an important role in influencing ileal fermentation by either providing specific substrates or shaping the ileal microbial composition to promote the production of specific organic acids.

Chapter 8: General discussion

This chapter presents the key findings of all the research presented, an additional discussion of the combined *in vivo/in vitro* ileal fermentation assay, an overall conclusion, and recommendations for future studies.

8.1. Key findings

The main aim of the research programme was to investigate the contribution of ileal fermentation to the overall GIT fermentation in adult humans. In addition, due to the challenge of collecting human ileal digesta, the validity of using the growing pig as an animal model for the adult human was tested. As hypothesised, the human ileal microbiota significantly contributed to the overall GIT fermentation, and the diet influenced this ileal fermentation. The research also established the growing pig as a valid model for studying human ileal fermentation.

Firstly, the combined *in vivo*/*in vitro* ileal fermentation methodology required validation in both the growing pig and adult human. Therefore, in **Chapter 3**, the *in vivo* disappearance of OM was compared with the *in vitro* ileal OM fermentation for the growing pig itself. In addition, whether the model could be improved by artificially rearing the pigs (i.e., in a more hygienic, non-farm environment and receiving a human-type diet) and inoculating them in early life with an infant faecal extract was tested. The rearing conditions were found to not affect the *in vivo* OM fermentability. There was agreement between the *in vivo* and *in vitro* OM fermentability for the control and artificially reared pigs but not for the artificially reared plus inoculated pigs. The rearing conditions did not affect the total number of ileal bacteria or the individual taxa. Only minor differences were observed in *in vitro* organic acid

production. Therefore, it is suggested that conventional pigs are the preferred model for studying ileal fermentation in humans. In addition, the *in vitro* ileal OM fermentability was similar to the *in vivo* OM fermentability in the pig, and therefore, the combined *in vivo*/*in vitro* ileal fermentation was found to be a valid model to determine *in vivo* ileal fermentation in the conventional pig.

The model was then validated for the adult human by fermenting *in vitro* different fibre substrates with inocula prepared from the ileal digesta of growing pigs and ileal effluent from human ileostomates (**Chapter 4**). The results showed that the ileal microbiota from human ileostomates was able to ferment (*in vitro*) different fibre sources (i.e., AG, FOS, and pectin) and provided a robust, reproducible *in vitro* model to study human ileal fermentation. Based on OM fermentability and organic acid production, similar fermentation of the fibre sources was observed when using ileal inocula from pigs and human ileostomates. In addition, the total number of bacteria and two-thirds of the bacterial taxa were similar between the ileal microbiota of pigs and human ileostomates. The results suggest that the growing pig can be used as a model for adult humans when studying ileal fermentation.

After the *in vivo*/*in vitro* ileal fermentation assay was validated, the first step was to determine the significance of this contribution and compare it with the hindgut fermentation. Ileal and hindgut fermentations in growing pigs fed a human-type diet for fourteen days were compared using the combined *in vivo*/*in vitro* fermentation assay (**Chapter 5**). A significant degree of OM fermentation was observed during ileal fermentation. Despite differences in the microbial community and fermentation time, the ileal OM fermentability was similar to the hindgut OM fermentability. Due to the amount of OM entering the ileum, the estimated amount of OM fermented in the ileum was actually greater than that in the hindgut. An important amount of SCFAs (mainly acetic acid) was produced during ileal fermentation. These SCFA were estimated to be absorbed or metabolised directly in the ileum.

For the work reported in **Chapter 6**, the objective was to test the effect of diet on the ileal microbiota and their fermentation and compare this with the hindgut fermentation. Growing pigs were fed three semi-synthetic diets containing cellulose (low overall GIT fermentability) as a sole fibre source (4.5%) or diets where half of the cellulose was replaced by psyllium or kiwifruit fibre (moderate overall GIT fermentability). This study was part of a larger study, which allowed small intestinal and caecal samples to be collected for this study. After 42 days, digesta were collected to perform *in vitro* fermentation. Firstly, this study confirmed the findings reported in Chapter 3 (i.e., a significant degree of ileal fermentation, which was comparable to hindgut fermentation). Secondly, partially replacing fibre with a low overall GIT fermentability (i.e., cellulose) with fibre with a higher overall GIT fermentability (i.e., psyllium and kiwifruit fibre) affected the OM fermentation, SCFA production, and the microbial community in both the ileum and the hindgut.

The effect of diet on the fermentative capacity of different ileal microbiota was also evaluated (**Chapter 7**). The inocula were obtained from ileal-cannulated pigs that received seven diets containing human foods with different dietary compositions for seven days as part of another study. The ileal microbiota were used to prepare inocula to ferment (*in vitro*) different fibre sources to test their fermentative capacity. The *in vitro* OM fermentability and the organic acid production were affected by both the microbiota in the inoculum and the available fibre source (i.e., substrate). However, the contribution of the fibre source was suggested to be the major contributor to ileal fermentation. In addition, specific bacterial taxa in the inoculum were linked to the OM fermentability and organic acid production of the specific fibre sources. These results suggest that dietary interventions could play an important role in influencing ileal fermentation.

8.2. *In vivo/in vitro* ileal fermentation assay

A combined *in vivo*/*in vitro* ileal fermentation assay that is a valid model for investigating human ileal fermentation has been established based on the overall research programme. However, the assay has some limitations. Firstly, the assay includes an *in vitro* ileal fermentation, which means that there is a potential fermentation of nutrients in the proximal ileal digesta (substrate) that may have been absorbed intact *in vivo*. In addition, the microbiota in the ileal inoculum used for the *in vivo* fermentation may differ from the microbiota in the ileal digesta due to the inoculum preparation. The anaerobic condition may favour the strict anaerobic microbiota during fermentation, which could affect the fermentation outcomes. However, as described in Chapter 3, *in vitro* and *in vivo* OM fermentability were in agreement. This finding suggests that even if the microbiota of the inoculum is different from that in the ileal digesta (not measured in this research), it did not affect the fermentation outcomes.

The combined *in vivo/in vitro* ileal fermentation assay was initially developed using an inoculum prepared by pooling ileal digesta from five pigs [267]. Pooled inocula have been used to standardize the microbiota within and across different experiments. This was presumed to give a more diverse microbial community which would be more representative of the microbiota of the whole study population. It had been found that a pooled faecal inoculum had a similar metabolic functionality in terms of SCFA production during *in vitro* faecal fermentation as the individual inocula of the same human subjects [376]. Nevertheless, in recent publications, individual inocula are used as the preferred option under the assumption that the microbial community is optimised with specific bacteria performing to function as a community within the GIT of an individual animal or human.

Therefore, when pooling multiple microbial communities together, a new equilibrium needs to be reached, resulting in specific bacteria performing different functions in the pooled inoculum due to functional redundancy. It should be noted that the research presented in Chapters 5, 6 and 7 was performed chronologically before that described in Chapters 3 and 4 as opportunities arose to collect ileal digesta samples from other studies. This is also the reason the experimental set up (such as the duration of the dietary intervention) differs between the chapters presented in the thesis. However, for future application of the combined *in vivo*/*in vitro* ileal fermentation, it is recommended to follow the set up described in Chapters 3 and 4 using individual inocula and a dietary intervention period of 14 days.

The ileal fermentation assay uses the growing pig as an animal model for the adult human. Pigs and humans were found to have somewhat different ileal microbial communities. Still, the OM fermentability and SCFA productions were similar between the two species (Chapter 4). This is partly explained by the results given in Chapter 7, where it was found that the substrate has a larger impact on the fermentation outcomes than the microbiota in the inoculum. However, another explanation is related to species redundancy within the microbial community, meaning that multiple bacterial species can perform a similar metabolic function within the microbial community. Taxonomic composition does not directly translate to fermentation outcomes. Therefore, measuring fermentation outcomes (such as OM fermentability and organic acid production) in addition to taxonomic composition when studying ileal fermentation is recommended.

During *in vitro* faecal fermentation, a correlation was found between the disappearance of dietary fibre and the production of SCFA [260]. In the current research, a direct relationship between the disappearance of OM and organic acid production was not found. For example, a relatively low OM fermentability of cellulose was observed with medium organic acid production (Chapter 7). It is unclear why there was no direct correlation, and more research

is warranted to determine the fermentation kinetics during *in vitro* ileal fermentation, including the nutrients consumed and metabolites formed.

Aside from the described limitations of the assay, it is a useful tool to provide a better understanding of human ileal fermentation. It has the advantage of allowing the measurement of fermentation outcomes (such as cumulative organic acid production) that would be challenging to measure *in vivo*.

8.3. Conclusion

In conclusion, the studies reported here demonstrated that the combined *in vivo*/*in vitro* ileal fermentation methodology is valid for predicting *in vivo* ileal fermentation in the growing pig itself and in the adult human, despite differences in microbial composition between the species. In addition, the research highlighted the contribution of ileal fermentation in terms of overall GIT fermentation. Ileal fermentation showed a significant degree of OM fermentability and organic acid production, similar to hindgut fermentation. Like hindgut fermentation, ileal fermentation can be influenced by diet. The research contributed to an understanding of the overall GIT fermentation and demonstrated the importance of ileal fermentation. In addition, it is established that dietary intervention may shape the ileal microbial community and produce specific organic acids that can be absorbed in the ileum to affect host health.

8.4. Future perspectives

The following aspects are recommended for future studies to strengthen the understanding of ileal fermentation and its effect on human health.

- This PhD thesis presents a valid (pig) model for studying human ileal fermentation. However, the model has been validated for only a limited number of foods or diets. From previous studies, it is known that food structure affects digestion and fermentation. Therefore, testing a wider variety of foods with different food structures is recommended to allow for a more comprehensive validation of the model.
- During the *in vitro* ileal fermentation, samples were only taken after two hours of fermentation. Taking samples at multiple time points during the *in vitro* ileal fermentation may provide insight into how substrates can be metabolised into organic acids over time and how the available substrate affects the microbiota in the inoculum over time.
- Investigating the disappearance of dietary fibre per se rather than focusing solely on OM. The disappearance of fibre could be done by determining, for example, the monomeric sugar composition or molecular sizes before and after *in vitro* ileal fermentation. Determining the fibre would allow testing as to whether the OM fermentation observed is actually due to the fibre breakdown and whether the bacteria in the ileal inoculum prefer specific monomers.
- 'Omics' technologies could be used to better understand the ileal microbiota and their fermentation. Metatranscriptomics, for example, would provide a further understanding of which genes are actively expressed in response to a dietary intervention (*in vivo*) or substrate availability (*in vitro*). In addition, metabolomics would allow the study of a wider variety of microbial metabolites formed during ileal fermentation.
- To test the effect of ileal fermentation products on GIT health, human epithelial cells (e.g., Caco-2 cells and ileal cell organoids) could be exposed to the supernatant after ileal fermentation. In addition to testing direct immune responses of the epithelial cells to microbial metabolites, it would also allow studying whether the microbial metabolites can cross the epithelial cell layer to support systemic function in the host.

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Appendix 1

Chapter 3: Supplementary material

Supplementary methods

In vivo experiment

Infant faecal extract. The collection of infant faecal material received ethical approval from the Massey University Human Ethics Committee Southern A, Palmerston North, New Zealand (protocol SOA 19/75). The six donor infants (less than six months old and mainly breastfed) had no reported diarrhoea or other digestive disorders and had received no antibiotics since birth. For each infant, one faecal sample obtained a maximum of four days before the start of the study was sent for diagnostic testing at Metlab Central (Palmerston North, New Zealand). All samples tested negative for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Escherichia coli* O157:H7 Rotavirus, and *Clostridium difficile* toxins A and B. The faeces from the infants were obtained fresh within 24 h before inoculation of the piglet and stored anaerobically (using the GasPak EZ pouch system, BD, New Jersey, U.S.) at -20 °C until used. The infant faecal extract was prepared on the day of administration by pooling faeces (n = 4 or 5 per inoculum) based on equal weight and diluted 20-fold in sterile 0.1 M potassium phosphate buffer (pH 7.2) [255] and kept on ice until needed.

Phase 2. The piglets were individually bowl-fed with a bovine-milk infant formula (Anmum NeoPro1, Fonterra NZ), prepared according to the manufacturer's instruction with added casein (0.6 g/100mL) to match the daily protein intake provided by the sow milk [295]. The infant formula was prepared fresh and warmed to 35°C in a water bath before feeding. The piglet's daily ration was 345 g liquid formula per kg of BW per day [296, 297]. The piglets were weighed upon arrival, then every third day until PND 14, and weekly after that. The

daily rations of the piglets were adjusted accordingly. From PND 7 to 13, the piglets received their daily ration across seventeen meals at hourly intervals from 0600 h to 2200 h. From PND 14 to 17, the daily ration was provided across seven meals at 2.5-hour intervals from 0630 h to 2130 h. From PND 18 to 28, the daily ration was provided as five meals at 3.5-hour intervals from 0630 h to 2030 h. At feeding time, a divider with four bowl slots was inserted into the cage to separate the piglets and allow individual feeding. The piglets were carefully observed (at least at every feeding time) to make sure the piglets were healthy. The health assessment included determining feed intake (at least 80% of their daily ration), body temperature, behavioural observation, faecal consistency, skin colouration, and signs of dehydration. Toys and towels were provided as enrichment to the piglets.

Chemical analysis

The organic acids were quantified in duplicate in the other half of the samples after *in vitro* fermentation (i.e., $n = 2$) using gas chromatography [302]. Standard curves were prepared using formate, acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, lactate, and succinate. 2-ethyl butyric acid (5 mM, Sigma Aldrich) was added as an internal standard to both the external standards (i.e., standard curve) and samples before derivatizing the organic acids with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (Sigma-Aldrich). Then, the organic acids in the samples and standards were quantified using a gas chromatograph (GC-2010, Shimadzu) with a DB-1MS UI (30 m x 0.25 mm x 0.25 µm, Agilent) connected to a flame ionization detector. Helium was the carrier gas. The organic acid concentrations in the fermentation samples were corrected by the concentrations found in the blank fermentations (i.e., the bottles with no added substrate, refer to the calculations in the Supplementary Methods). Every time

sample preparation and GC analysis were done, a representative control sample was included to test the method's performance. The amounts of butyric, valeric, iso-butyric, and iso-valeric acid detected in the samples were negligible (i.e., below the detection limit). Therefore, the total organic acid production was defined as the sum of formic, acetic, propionic, lactic, and succinic acid concentrations.

Microbial analysis

qPCR analysis. The total number of bacteria (i.e., 16S rRNA gene copies) was determined in the DNA extracted for the ileal samples and the standards (i.e., DNA from *Escherichia coli,* strain ATCC 8739, at the concentration of 10^2 to 10^{10} gene copies/µL) in duplicate using a quantitative PCR (qPCR). This method used the PowerTrack SYBR Green Master Mix (Thermo Fisher) according to the manufacturer's instructions, after which the samples and standards were loaded onto a Quant Studio™ 3 System (Applied Biosystems). The forward primer used was 5'-TCCTACGGGAGGCAGCAGT, and the reverse primer was (5'-GGACTACCAGGGTATCTAATCCTGTT [333]. Both primers were obtained from Integrated DNA Technologies. Every qPCR run included an activation cycle (95°C, 5 min), 40 run cycles (i.e., denaturation (95°C, 30 s), annealing 60°C, 60 s) and extension (72°C, 60 s)) and one melting curve (60–95°C at 0·1°C/s) [286]. The total number of 16S rRNA gene copies in the ileal digesta and the relative abundance of the taxa were used to determine the number of 16S rRNA gene copies per taxa with the assumption that each taxon has an equal number of 16s rRNA gene copies (calculation in Supplementary methods).

16S rRNA gene sequencing and bioinformatics. Extracted DNA samples were sent to the Massey Genome Service (Massey University) for Illumina MiSeq sequencing to determine the taxonomic composition [286]. To amplify the V3-V4 hypervariable region of the 16S

rRNA gene, the following primers were used: 16SF_V3 (5' - AATGATACGGCGACCACCGAGATCTACAC-index- TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index- AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). 16SF_V3 (5' - AATGATACGGCGACCACCGAGATCTACAC-index- TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index- AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). The 96 libraries will be prepared using the Illumina 16S V3-V4 rRNA library preparation method [334]. The Massey Genome Service has dual index PCR primers which flank the V3-V4 hyper-variable region of 16S rRNA, which uses a Single Step PCR Library preparation method to prepare the libraries. The libraries were run on an Illumina MiSeq™ 2X 250 base PE, version 2 chemistry.

The bioinformatics analysis was done using Mothur V1.46.1 (2, 3). Briefly, 3,707,642 paired-end reads were detected. These reads were assembled and underwent quality control, removing all reads with over eight homopolymers and uncalled bases. The average length of the sequences was 420 bp. The SILVA database (version 138) (4) was used to align the sequences. After alignment, sequences were pre-clustered (4 bp) to remove noise and reduce the effect of sequencing errors. Chimeras were removed using VSEARCH (6), and all non-bacterial sequences were excluded. The remaining 3,208,212 reads were clustered into OTUs with a 97% cut off. The resulting BIOM table was used as input for Microbiome analyst [304, 305]. MicrobiomeAnalyst provided the taxonomic composition and Shannon diversity numbers and performed the principal coordinates analysis (PCoA) with the Bray-Curtis dissimilarities.

A second bioinformatic analysis was performed using QIIME [335] to predict metabolic functionality similar to that described above. Data were aligned with the SILVA database (version 138) (4). The resulting BIOM table was used as input for MicrobiomeAnalyst [304, 305] to get the predicted metabolic functionality (i.e., KEGG pathways) using the Tax4Fun [377].

Calculations

To determine the number of bacteria per taxa in the ileal digesta, it was assumed that each taxon had an equal number of 16S rRNA gene copies. The number of bacteria per taxa in ileal digesta was calculated as follows [286]:

1) Number of bacteria per $taxa_{ileal\ digesta}$ (16S rRNA gene copy number/g digesta) = total number of $bacteria_{ileal\ digesta}$ (16S rRNA gene copy number/g digesta) x relative $abundance_{\ taxa}$ (%) / 100

The *in vivo* ileal OM fermentability was calculated with the normalised OM content in the proximal and terminal ileal digesta [292]:

2) Normalised OM $content_{proximal\ or\ terminal\ ileum}$ (g/kg diet DM intake) = $OM_{proximal\ or\ terminal\ ileal\ digesta}$ (g/kg DM) x ($TiO_{2\text{-diet}}$/$TiO_{2\text{-proximal or ileal digesta}}$)
3) OM $fermentability_{in\ vivo}$ (%) = (normalised OM $content_{proximal\ ileum}$ (g/ kg diet DM intake) – normalised OM $content_{terminal\ ileum}$ (g/ kg diet DM intake) / normalised OM $content_{proximal\ ileum}$ (g/ kg diet DM intake) x 100

The following equations were used to determine *in vitro* ileal OM fermentability [292]:

4) OM $fermentability_{in\ vitro}$ (%) = (OM $_{before\ fermentation}$ – [OM $_{after\ fermentation}$ – ((OM $_{blank\ initial}$ + OM $_{blank\ final}$)/2)]) / OM $_{before\ fermentation}$ x 100

where $OM_{blank\ initial}$ and $OM_{blank\ final}$ are the amounts of OM in the blanks before (initial) and after (final) *in vitro* fermentation, respectively. The *in vitro* ileal fermentation had its own blanks for each individual pig.

The *in vitro* ileal production of organic acids was determined as described previously [292] using the following equations:

5) Organic acid production (mmol/kg substrate DM incubated) = (organic acid $_{after\ fermentation}$ (mmol) – [(organic acid $_{blank\ initial}$ + organic acid $_{blank\ final}$)/2]) / kg DM substrate

where organic acid $_{blank\ initial}$ and organic acid $_{blank\ final}$ are the organic acids (mmol) in the blanks before (initial) and after (final) *in vitro* fermentation, respectively. The *in vitro* ileal fermentation had its own blanks for each individual pig.

Supplementary tables

Supplementary Table 3.1: The ingredient and determined nutrient composition of the human-type diet given as three different meals (i.e., breakfast, lunch, and dinner) per day to the AR and AR+ pigs in Phase 3 of the study[1]

	Human-type diet			
	Breakfast	**Lunch**	**Dinner A**	**Dinner B**
Ingredient, g/kg DM				
Milk (UHT, 3.3% fat)	304	-	-	-
Wheat biscuits (Weet-bix, Sanitarium, crushed)	468	-	-	-
Banana (ripened, peeled)	203	-	-	-
Wheat bread (white, sliced)	-	570	-	-
Cheese (cheddar, grated)	-	264	-	-
Apple (raw, minced)	-	141	-	-
Egg (boiled, minced)	-	-	335	206
Baked beans (canned, minced)	-	-	241	241
Fruit salad (canned, minced)	-	-	98	98
Corn (canned)	-	-	104	104
Rice (white, cooked)	-	-	197	329
Premix of vitamins and minerals[2]	5.0	5.0	5.0	5.0
Titanium dioxide	3.0	3.0	3.0	3.0
Limestone	2.0	2.0	2.0	2.0
Di-calcium phosphate	15	15	15	15
Nutrient, g/kg DM				
Ash	64.1	59.7	68.5	63.0
Crude protein	180	194	261	218
Total lipids	75.6	157	132	88.3
Starch	320	378	245	348
Total dietary fibre	118	59.0	100	153
- Insoluble fibre	88.8	42.3	84.5	134
- Soluble fibre	29.0	16.7	15.7	19.5
Gross energy MJ/kg	201	218	223	210

[1]The formulation of the diets was based on the chemical composition of the ingredients obtained from the New Zealand Food Composition Database (https://www.foodcomposition.co.nz/) and to meet the requirements of starting pigs as prescribed by the National Research Council, 2012 [298]; Dinner A and B were formulated to provide the different nutrient requirements for pigs of 7-11 and 11-25 kg bodyweight, respectively; DM, dry matter, UHT, ultra-high temperature.

[2]The vitamin and mineral premix (Pig Creep/Weaner Premix High copper, Nutritech, Auckland, New Zealand) supplied (per kg DM diet): vitamin A, 4.2 µg; vitamin D3, 0.075 µg; vitamin E, 47 mg; vitamin K3, 4 mg; thiamine, 3 mg; riboflavin, 6 mg; pyridoxin, 4 mg; vitamin B12, 30 µg; Biotin, 40 µg; Niacin, 30 mg; Pantothenic Acid, 22 mg; Folic Acid, 0.5 mg; Choline, 180 mg, Cobalt, 2 mg; Copper 250 mg; Iodine, 2 mg; Iron 160 mg; Manganese, 60 mg, Selenium, 0.6 mg; Zinc, 230 mg.

Supplementary Table 3.2: Effect of rearing regimen on the number of bacteria in ileal digesta pigs reared under different environmental conditions and fed a human-type diet for the last fourteen experimental days[1]

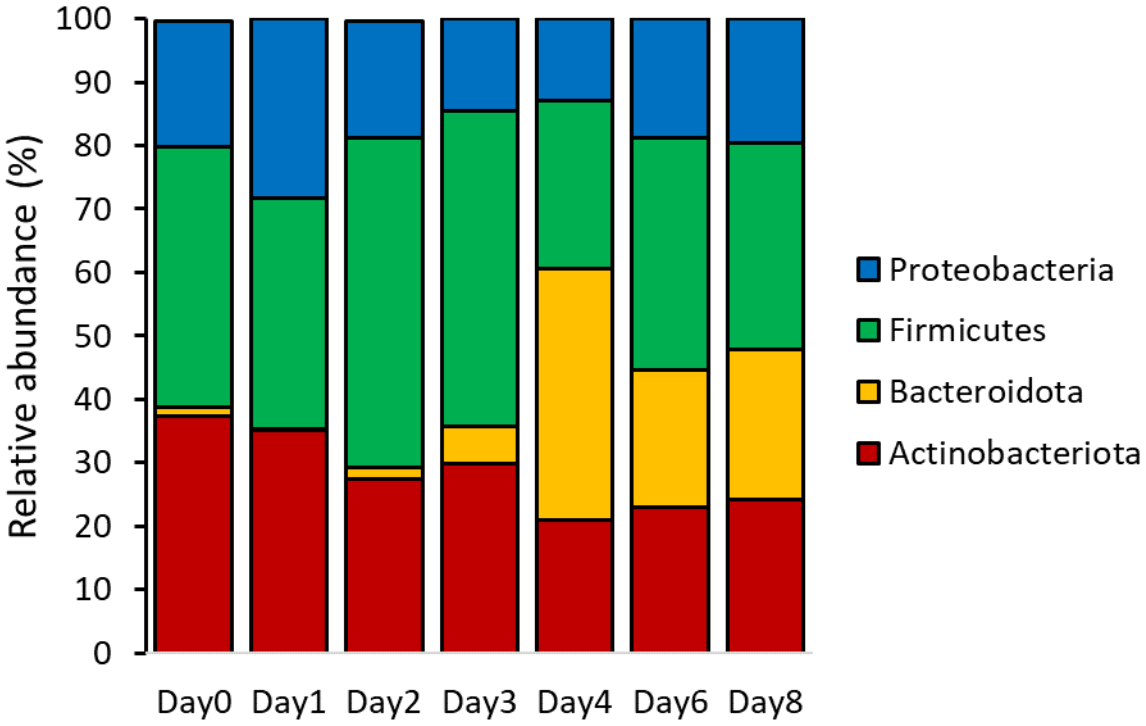
Phylum	Genus	Rearing regimen			*P* value
		AR	AR+	Control	
		Log_{10} 16S rRNA gene copies/g digesta			
Total bacteria		9.33 ± 0.259	9.63 ± 0.190	9.80 ± 0.111	0.232
Actinobacteria		7.04 ± 0.192[b]	7.51 ± 0.192[a]	7.42 ± 0.192[ab]	0.033
	Actinomyces	6.43 ± 0.281[b]	6.97 ± 0.224[a]	6.48 ± 0.271[b]	0.011
	Bifidobacterium	4.20 ± 0.422[b]	4.58 ± 0.351[ab]	5.45 ± 0.390[a]	0.020
	Corynebacterium	5.77 ± 0.244	5.62 ± 0.253	6.04 ± 0.078	0.222
	Kocuria	4.83 ± 0.354	5.30 ± 0.152	5.30 ± 0.371	0.449
	Rothia	6.77 ± 0.207[b]	7.23 ± 0.169[a]	6.88 ± 0.221[ab]	0.031
Bacteroidetes		6.38 ± 0.194	6.48 ± 0.135	6.58 ± 0.140	0.593
	Bacteroides	6.17 ± 0.181	6.15 ± 0.146	6.28 ± 0.136	0.597
Firmicutes		9.29 ± 0.252	9.61 ± 0.192	9.74 ± 0.120	0.251
	Aerococcus	4.93 ± 0.244	4.99 ± 0.382	5.31 ± 0.376	0.580
	Bacillus	5.36 ± 0.218	5.09 ± 0.293	5.36 ± 0.264	0.475
	Blautia	5.01 ± 0.260	5.24 ± 0.279	5.25 ± 0.269	0.622
	Carnobacterium	6.03 ± 0.220	5.90 ± 0.279	5.88 ± 0.313	0.654
	Cellulosilyticum	4.75 ± 0.467	4.66 ± 0.415	4.27 ± 0.214	0.525
	Clostridiaceae_unclassified	6.11 ± 0.310	6.46 ± 0.361	6.68 ± 0.216	0.273
	Clostridium_sensu_stricto_1	8.57 ± 0.314	8.93 ± 0.350	9.21 ± 0.207	0.179
	Clostridium_sensu_stricto_13	6.00 ± 0.223	5.98 ± 0.276	5.70 ± 0.233	0.200
	Enterococcus	6.42 ± 0.380	7.00 ± 0.399	7.19 ± 0.469	0.074
	Gemella	5.60 ± 0.206	5.87 ± 0.173	5.86 ± 0.309	0.324

Lachnospiraceae_unclassified	5.52 ± 0.230[ab]	5.87 ± 0.209[a]	5.37 ± 0.198[b]	0.045
Lactobacillus	7.82 ± 0.343	8.50 ± 0.280	7.96 ± 0.306	0.225
Lactococcus	6.37 ± 0.124	6.59 ± 0.205	6.47 ± 0.084	0.625
Leuconostoc	8.44 ± 0.381	8.66 ± 0.335	8.68 ± 0.376	0.561
Ligilactobacillus	6.20 ± 0.189	6.70 ± 0.259	6.59 ± 0.233	0.249
Limosilactobacillus	7.77 ± 0.463[ab]	8.43 ± 0.299[a]	7.37 ± 0.352[b]	0.029
Macrococcus	4.26 ± 0.331	4.42 ± 0.241	4.85 ± 0.378	0.505
Megasphaera	3.47 ± 0.247	ND	4.31 ± 0.417	0.075
Mycoplasma	ND	ND	7.46 ± 0.244	-
Romboutsia	8.17 ± 0.345	8.58 ± 0.390	8.39 ± 0.227	0.729
Ruminococcaceae_unclassified	4.16 ± 0.407	4.66 ± 0.262	4.02 ± 0.202	0.180
Staphylococcus	5.71 ± 0.172	5.77 ± 0.228	6.08 ± 0.082	0.128
Streptococcus	7.24 ± 0.244[b]	7.36 ± 0.182[b]	8.29 ± 0.224[a]	0.001
Terrisporobacter	6.16 ± 0.491[b]	6.75 ± 0.465[ab]	7.98 ± 0.465[a]	0.025
Turicibacter	7.96 ± 0.430	8.25 ± 0.486	8.24 ± 0.318	0.854
Proteobacteria	7.73 ± 0.328	7.62 ± 0.217	8.28 ± 0.237	0.136
Actinobacillus	4.00 ± 0.533	4.68 ± 0.469	4.39 ± 0.675	0.444
Burkholderia_Caballeronia_Paraburkholderia	6.28 ± 0.189	6.34 ± 0.184	6.19 ± 0.203	0.709
Citrobacter	4.94 ± 0.575	5.15 ± 0.505	5.99 ± 0.380	0.237
Enhydrobacter	4.89 ± 0.323	5.53 ± 0.221	5.69 ± 0.366	0.204
Enterobacteriaceae_unclassified	4.34 ± 0.563	5.07 ± 0.433	5.81 ± 0.378	0.110
Escherichia-Shigella	6.89 ± 0.442	6.86 ± 0.307	7.80 ± 0.339	0.111
Klebsiella	4.99 ± 0.643	5.44 ± 0.589	6.23 ± 0.464	0.248
Pasteurellaceae_unclassified	ND	3.97 ± 0.327	4.41 ± 0.385	0.389
Pseudomonas	7.03 ± 0.191	6.97 ± 0.150	7.27 ± 0.117	0.273
Serratia	5.62 ± 0.203	5.25 ± 0.210	5.18 ± 0.287	0.150

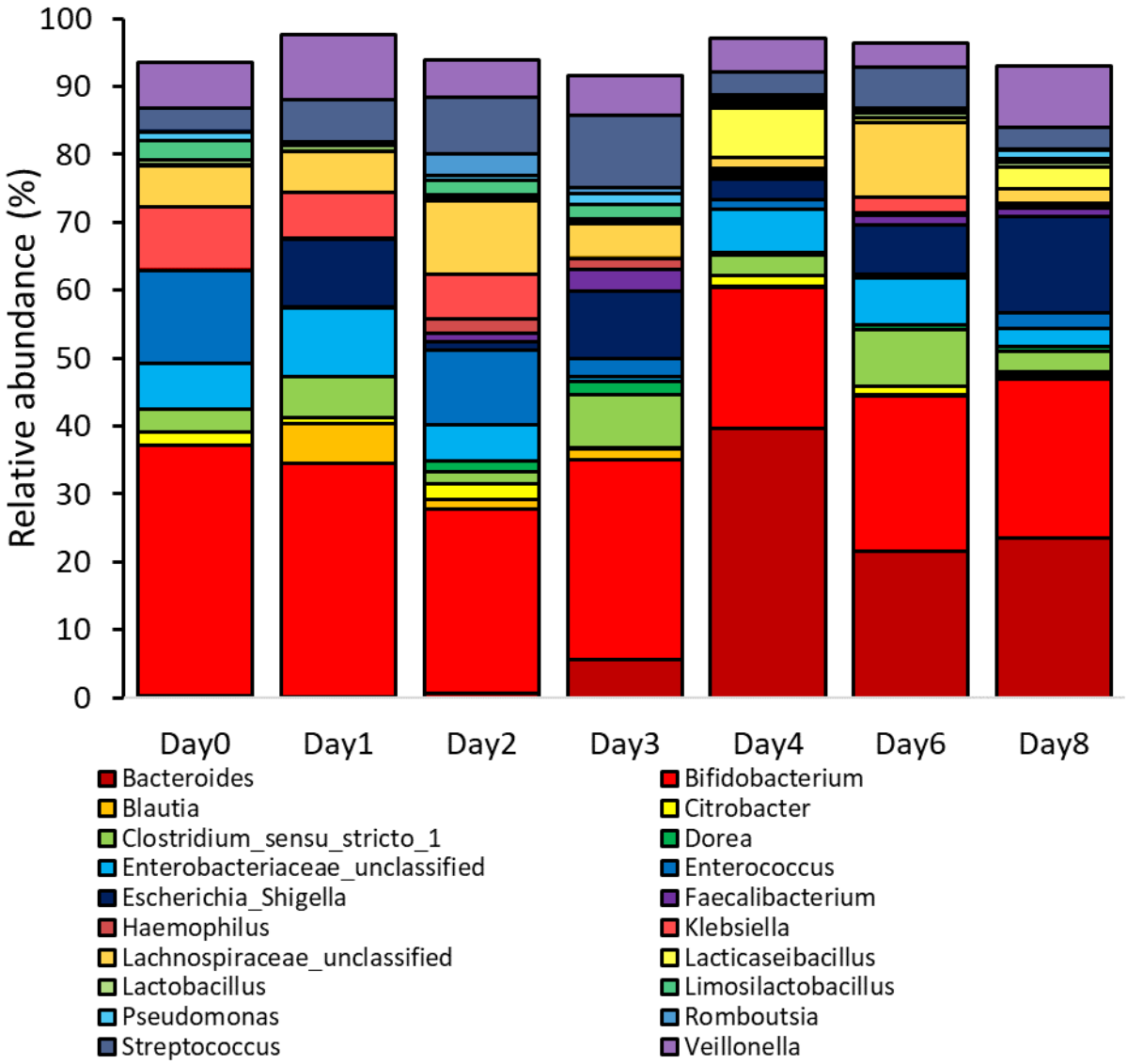
Yersinia	5.46 ± 0.227	5.69 ± 0.260	5.29 ± 0.248	0.235
Yersiniaceae_unclassified	5.96 ± 0.194	6.02 ± 0.188	6.35 ± 0.278	0.388
Verrucomicrobiota	ND	4.26 ± 0.469	4.31 ± 0.253	0.937
Chlamydia	ND	4.15 ± 0.475	4.12 ± 0.261	0.959

[1] Values are means ± SEM, n = 10 per treatment. No significant difference was observed in the DM content of the ileal digesta between the different rearing regimens; therefore, the data is present on a wet digesta basis. Only taxa with >0.1% relative abundance in at least one sample are presented. The number of 16S rRNA gene copies per taxa was obtained by multiplying the total number of 16S rRNA gene copies with the relative abundance of the taxa with the assumption that each taxon has an equal number of 16S rRNA gene copies. Data were $\log_{10}$ transformed to achieve homogenous variance. A one-way ANOVA model was used to assess the effect of the rearing regimen. Means in a row with a different letter differ (P < 0.05). AR, artificially reared; AR+, artificially reared plus inoculated with infant faecal extract; ND, not detected.

Supplementary figures



Supplementary Figure 3.1: The relative abundance (%) of bacterial phyla found in the infant faecal extract used to inoculate piglets. Each inoculum was prepared by pooling fresh faeces obtained from four or five infants. Only phyla with >0.5% relative abundance in at least one sample are presented.



Supplementary Figure 3.2: Relative abundance (%) of the twenty most abundant bacterial genera found in the infant faecal extract used to inoculate piglets. Each inoculum was prepared by pooling fresh faeces obtained from four or five infants.

Appendix 2

Chapter 4: Supplementary material

Supplemental methods

Microbial analysis

qPCR analysis. After DNA extraction, the total number of bacteria (i.e., 16S rRNA gene copies) in the ileal digesta samples obtained from pigs and humans were determined in duplicate using a quantitative PCR (qPCR). A standard curve was made using purified DNA from *Escherichia coli* ATCC 8739 and had a range of 10^2 to 10^{10} gene copies/µL. This method used the PowerTrack SYBR Green Master Mix (Thermo Fisher) according to the manufacturer's instructions, after which the samples and standards were loaded onto a Quant Studio™ 3 System (Applied Biosystems). The forward primer used was 5'-TCCTACGGGAGGCAGCAGT, and the reverse primer was (5'-GGACTACCAGGGTATCTAATCCTGTT [333]. Both primers were obtained from Integrated DNA Technologies. Every qPCR run included an activation cycle (95°C, 5 min), 40 run cycles (i.e., denaturation (95°C, 30 s), annealing 60°C, 60 s) and extension (72°C, 60 s)) and one melting curve (60–95°C at 0·1°C/s) [286]. The total number of 16S rRNA gene copies in the ileal digesta and the relative abundance of the taxa were used to determine the number of 16S rRNA gene copies per taxa with the assumption that each taxon has an equal number of 16s rRNA gene copies (calculation in Supplementary methods).

16S rRNA gene sequencing and bioinformatics. Extracted DNA samples were sent to the Massey Genome Service (Massey University) for Illumina MiSeq sequencing to determine the taxonomic composition [286]. To amplify the V3-V4 hypervariable region of the 16S rRNA gene, the following primers were used: 16SF_V3 (5' -

AATGATACGGCGACCACCGAGATCTACAC-index-TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). 16SF_V3 (5’ - AATGATACGGCGACCACCGAGATCTACAC-index-TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5’ - CAAGCAGAAGACGGCATACGAGAT-index-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). The 96 libraries will be prepared using the Illumina 16S V3-V4 rRNA library preparation method [334]. The Massey Genome Service has dual index PCR primers which flank the V3-V4 hyper-variable region of 16S rRNA, which uses a Single Step PCR Library preparation method to prepare the libraries. The libraries were run on an Illumina MiSeq™ 2X 250 base PE, version 2 chemistry.

The bioinformatics analysis was done using Mothur V1.46.1 (2, 3) and is described in Chapter 3. A total of 3,707,642 paired-end reads were detected. These reads were assembled and underwent quality control removing all reads with more than eight homopolymers and uncalled base. The average length of the sequences was 420 bp. The SILVA database (version 138) (4) was used to align the sequences. After alignment, sequences were pre-clustered (4 bp) to remove noise and reduce the effect of sequencing errors. Chimeras were removed using VSEARCH (6), and all non-bacterial sequences were excluded. The remaining 3,208,212 reads were clustered into OTUs with a 97% cut-off. The resulting BIOM table was used as input for MicrobiomeAnalyst [304, 305]. MicrobiomeAnalyst provided the taxonomic composition and alpha diversity (i.e., Chao1 and Shannon Diversity Indexes) and performed the principal coordinates analysis (PCoA) with the Bray-Curtis dissimilarities.

A second bioinformatic analysis was performed using QIIME [335] to obtain the predicted metabolic functionality similar to that described above. Data were aligned with the SILVA database (version 138) (4). The resulting BIOM table was used as input for MicrobiomeAnalyst [304, 305] to obtain the predicted metabolic functionality (i.e., KEGG pathways) using the Tax4Fun [377].

Calculations

To determine the number of bacteria per taxa in ileal digesta and ileal effluent, it was assumed that each taxon had an equal number of 16S rRNA gene copies. The number of bacteria per taxa in ileal digesta was calculated as follows [286]:

1) Number of bacteria per $taxa_{ileal\ digesta}$ (16S rRNA gene copy number/g digesta) = total number of $bacteria_{ileal\ digesta}$ (16S rRNA gene copy number/g digesta) x relative $abundance_{taxa}$ (%) / 100

The *in vitro* ileal OM fermentability was calculated as follows [292]:

2) $OM\ fermentability_{in\ vitro}$ (%) = ($OM_{before\ fermentation}$ – [$OM_{after\ fermentation}$ – (($OM_{blank\ initial}$ + $OM_{blank\ final}$)/2)]) / $OM_{before\ fermentation}$ x 100

where $OM_{blank\ initial}$ and $OM_{blank\ final}$ are the amounts of OM in the blanks before (initial) and after (final) *in vitro* fermentation, respectively. Blanks were included in the *in vitro* fermentation for each pig or human ileostomy subject.

The *in vitro* ileal production of organic acids was determined as described previously [292]:

3) Organic acid production (mmol/kg substrate DM incubated) = ($organic\ acid_{after\ fermentation}$ (mmol) – [($organic\ acid_{blank\ initial}$ + $organic\ acid_{blank\ final}$)/2]) / kg DM substrate

where organic acid $_{\text{blank initial}}$ and organic acid $_{\text{blank final}}$ are the organic acids (mmol) in the blanks before (initial) and after (final) *in vitro* fermentation, respectively. Blanks were included in the *in vitro* fermentation for each pig or human ileostomy subject.

Supplementary tables

Supplementary Table 4.1: The ingredient and determined nutrient composition of the experimental diet given to the pigs (Chapter 3)[1]

	Amount
Ingredient, g/kg DM	
Milk (UHT, 3.3% fat)	130
Wheat biscuits (Weet-Bix, Sanitarium, Auckland, NZ; crushed)	183
Apple (Braeburn; raw, minced)	122
Egg (boiled, minced)	67
Baked beans (Wattie's, Hastings NZ; canned, minced)	96
Rice (white, cooked)	378
Premix of vitamins and minerals[2]	5.0
Titanium dioxide	3.0
Limestone	10
Dicalcium phosphate	5.0
Nutrient, g/kg DM	
Ash	50
Crude protein	180
Total fat	107
Starch	374
Total dietary fibre	130
- Insoluble fibre	101
- Soluble fibre	29
Gross energy, MJ/kg	208

[1]The formulation of the diets was based on the chemical composition of the ingredients obtained from the New Zealand Food Composition Database (https://www.foodcomposition.co.nz/) and to meet the requirements of growing pigs as prescribed by the National Research Council, 2012 [298]. DM, dry matter; UHT, ultra-high temperature.

[2]The vitamin and mineral premix (Pig Grower/Finisher Premix High copper, Nutritech, Auckland, New Zealand) supplied (per kg DM diet): vitamin A, 4.2 µg; vitamin D3, 0.075 µg; vitamin E, 47 mg; vitamin K3, 4 mg; thiamine, 3 mg; riboflavin, 6 mg; pyridoxin, 4 mg; vitamin B12, 30 µg; Biotin, 40 µg; Niacin, 30 mg; Pantothenic Acid, 22 mg; Folic Acid, 0.5 mg; Choline, 180 mg, Cobalt, 2 mg; Copper 250 mg; Iodine, 2 mg; Iron 160 mg; Manganese, 60 mg, Selenium, 0.6 mg; Zinc 230 mg.

Supplementary Table 4.2: Taxonomic composition of ileal digesta from growing pigs and ileal effluent from human ileostomates that received a similar diet[1]

Phylum	Genus	Pig	Human	*P* value
		Log_{10} 16S rRNA gene copies/g DM digesta or effluent		
Total bacteria		9.80 ± 0.100	8.29 ± 0.854	0.153
Actinobacteria		7.45 ± 0.121	7.17 ± 0.798	0.750
	Actinomyces	6.54 ± 0.154	6.69 ± 0.676	0.836
	Actinomycetaceae_unclassified	ND	4.99 ± 0.710	-
	Atopobium	4.70 ± 0.245	5.40 ± 0.867	0.476
	Bifidobacterium	5.34 ± 0.375	5.75 ± 1.17	0.754
	Collinsella	5.07 ± 0.338	4.24 ± 1.07	0.496
	Coriobacteriaceae_UCG_002	4.25 ± 0.217	4.37 ± 1.06	0.914
	Corynebacterium	6.04 ± 0.078	5.14 ± 0.620	0.221
	F0332	ND	5.12 ± 0.717	-
	Kocuria	5.22 ± 0.397	4.18 ± 0.811	0.213
	Olsenella	4.49 ± 0.217	4.76 ± 0.734	0.741
	Rothia	6.94 ± 0.169	6.24 ± 0.699	0.380
	Scardovia	ND	4.23 ± 1.12	-
Bacteroidota		6.59 ± 0.133	6.05 ± 1.06	0.636
	Bacteroides	6.27 ± 0.093	5.49 ± 1.38	0.602
	Chryseobacterium	4.65 ± 0.317	3.35 ± 1.02	0.279
	Muribaculaceae_ge	5.21 ± 0.246	4.95 ± 1.24	0.843
	Prevotellaceae_unclassified	4.38 ± 0.189	4.25 ± 1.11	0.915
Firmicutes		9.74 ± 0.102	8.13 ± 0.834	0.124
	Abiotrophia	ND	4.49 ± 0.811	-
	Aerococcus	5.33 ± 0.363	ND	-
	Allobaculum	4.99 ± 0.248	4.60 ± 1.12	0.750
	Anaerovoracaceae_ge	4.23 ± 0.247	5.24 ± 0.636	0.095
	Bacillus	5.35 ± 0.182	4.89 ± 0.758	0.588
	Blautia	5.28 ± 0.235	4.55 ± 1.12	0.554
	Carnobacterium	5.87 ± 0.261	5.33 ± 1.25	0.690
	Clostridiaceae_unclassified	6.68 ± 0.186	3.85 ± 0.797	0.022
	Clostridium_sensu_stricto_1	9.20 ± 0.177	5.19 ± 0.766	0.005
	Clostridium_sensu_stricto_13	5.70 ± 0.138	5.37 ± 1.28	0.813
	Enterococcus	7.26 ± 0.191	5.31 ± 0.605	0.029
	Faecalibacterium	3.93 ± 0.142	3.75 ± 0.867	0.850
	Fusicatenibacter	4.01 ± 0.177	3.52 ± 1.12	0.686
	Gemella	5.89 ± 0.226	6.08 ± 0.624	0.732

Granulicatella	4.24 ± 0.276	6.16 ± 0.531	0.003
Intestinibacter	5.34 ± 0.307	3.51 ± 0.901	0.030
Lachnoanaerobaculum	4.23 ± 0.161	4.84 ± 0.561	0.345
Lachnoclostridium	4.11 ± 0.155	3.76 ± 0.922	0.733
Lachnospiraceae_unclassified	5.40 ± 0.182	5.21 ± 0.766	0.815
Lactobacillus	7.97 ± 0.296	5.54 ± 0.761	0.003
Lactococcus	6.48 ± 0.081	5.39 ± 0.828	0.262
Leuconostoc	8.78 ± 0.200	3.93 ± 0.883	0.004
Ligilactobacillus	6.59 ± 0.233	5.35 ± 1.57	0.478
Limosilactobacillus	7.41 ± 0.318	5.49 ± 1.22	0.194
Macrococcus	4.85 ± 0.378	ND	-
Megasphaera	4.31 ± 0.419	2.74 ± 0.950	0.099
Mycoplasma	7.46 ± 0.244	ND	-
Oribacterium	3.91 ± 0.082	5.36 ± 0.693	0.103
Parvimonas	4.54 ± 0.193	5.04 ± 0.727	0.539
Peptostreptococcus	5.34 ± 0.241	5.63 ± 0.615	0.597
Romboutsia	8.39 ± 0.227	5.47 ± 0.880	0.027
Solobacterium	ND	4.86 ± 0.584	-
Staphylococcus	6.08 ± 0.082	5.60 ± 0.692	0.524
Stomatobaculum	ND	4.95 ± 0.742	-
Streptococcus	8.29 ± 0.156	7.70 ± 0.722	0.459
Terrisporobacter	7.96 ± 0.473	4.20 ± 0.794	0.001
Turicibacter	8.23 ± 0.324	4.83 ± 0.741	<0.001
Veillonella	4.47 ± 0.236	6.90 ± 0.843	0.042
Veillonellaceae_unclassified	ND	4.83 ± 1.14	-
Fusobacteriota	5.01 ± 0.228	5.15 ± 0.824	0.879
Fusobacterium	5.00 ± 0.225	5.10 ± 0.839	0.916
Patescibacteria	5.46 ± 0.205	5.58 ± 0.717	0.882
Candidatus_Saccharimonas	ND	4.08 ± 0.686	-
Saccharimonadaceae_ge	4.16 ± 0.179	4.77 ± 1.15	0.629
Saccharimonadales_unclassified	5.34 ± 0.221	3.32 ± 0.658	0.003
TM7x	4.02 ± 0.158	4.97 ± 0.476	0.033
Proteobacteria	8.28 ± 0.237	7.49 ± 0.989	0.480
Actinobacillus	4.54 ± 0.535	2.94 ± 0.921	0.132
Burkholderia_Caballeronia_Paraburkholderia	6.19 ± 0.161	5.66 ± 0.615	0.442
Citrobacter	5.99 ± 0.381	4.51 ± 0.720	0.064
Enhydrobacter	5.69 ± 0.366	3.95 ± 1.04	0.070
Enterobacteriaceae_unclassified	5.82 ± 0.381	5.00 ± 0.736	0.290
Escherichia-Shigella	7.80 ± 0.339	6.64 ± 1.03	0.194

Haemophilus	ND	6.30 ± 1.14	-
Klebsiella	6.27 ± 0.440	4.11 ± 1.27	0.064
Pasteurellaceae_unclassified	4.41 ± 0.385	3.82 ± 1.11	0.537
Pseudomonas	7.27 ± 0.117	6.00 ± 1.44	0.430
Rahnella1	4.37 ± 0.275	4.37 ± 1.04	0.995
Serratia	5.23 ± 0.240	4.81 ± 1.14	0.736
Shewanella	4.73 ± 0.273	4.46 ± 1.08	0.818
Yersinia	5.31 ± 0.198	4.94 ± 1.18	0.770
Yersiniaceae_unclassified	6.34 ± 0.177	5.20 ± 1.22	0.405

[1] Values are means ± SEM; *n* = 10 pigs and 5 humans. The taxa shown in the table had a minimal relative abundance of 0.1% in at least one of the samples. To calculate the number of 16S rRNA gene copies per taxa, the total number of 16S rRNA gene copies was multiplied by the relative abundance of the taxa, assuming an equal number of 16S rRNA gene copies per taxon. A $\log_{10}$ transformation of the data was needed to achieve homogenous variance. The effect of species was evaluated using an independent Student's *t* test. DM, dry matter; ND, not detected.

Appendix 3

Chapter 5: Supplementary material

Supplementary tables

Supplementary Table 5.1: Ingredient and determined nutrient composition of the experimental diet[1]

	Amount
Ingredient, *g DM/kg DM*	
Cooked carrot (minced)	100
Canned beans (minced)	150
Peeled hard-boiled egg (minced)	120
Cooked white rice (minced)	550
Raw apple (minced)	48.5
Premix of vitamins and minerals[2]	5.0
Sodium chloride	3.0
Titanium dioxide	3.0
Calcium carbonate	0.5
Dicalcium phosphate	20
Nutrient, *g/kg DM*	
Crude protein	143
Starch	508
Total lipid	45
Ash	49
Soluble fibre	20
Insoluble fibre	96
Total dietary fibre	116
Determined energy, MJ/kg DM	
Gross energy	18.1

[1]The chemical composition of the ingredients was obtained from the USDA National Nutrient Database (https://ndb.nal.usda.gov/; version 2.3.8). DM, dry matter.

[2]Vitamin and mineral premixes were obtained from Vitec Nutrition Ltd (Auckland, New Zealand) and supplied (per kg of diet): Mn, 45 mg; Zn, 80 mg; Cu, 25 mg; Co, 0.5 mg; Se, 0.3 mg; Fe, 100 mg; Iodine, 1.0 mg; Choline, 100 mg; all-trans retinylacetate, 3.0 mg; cholecalciferol, 0.05 mg; α-tocopherol, 50 mg; menadione, 2.0 mg; thiamine, 1.0 mg; riboflavin, 3.0 mg; nicotinic acid, 15 mg; pantothenic acid, 20 mg; pyridoxine, 2.0 mg; cyanocobalamin, 0.01 mg; folic acid, 0.5 mg; biotin, 0.1 mg.

Supplementary Table 5.2: Predicted metabolic activity for carbohydrate and protein metabolism in ileal and caecal digesta of pigs fed a human-type diet, based on PICRUSt analysis[1]

KEGG reference pathway	GIT location		*P* value
	Ileum	Caecum	
	x10^6 relative activity/kg diet DM intake		
Carbohydrate metabolism			
Amino sugar and nucleotide sugar metabolism	113 ± 13.8	108 ± 8.03	0.635
Ascorbate and aldarate metabolism	16.2 ± 1.83	9.06 ± 0.67	0.023
Butanoate metabolism	49.8 ± 5.07	49.4 ± 3.41	0.919
C5-Branched dibasic acid metabolism	17.1 ± 2.76	25.3 ± 2.16	0.009
Citrate cycle (TCA cycle)	30.6 ± 2.48	43.8 ± 3.62	0.006
Fructose and mannose metabolism	67.0 ± 3.93	82.4 ± 6.62	0.053
Galactose metabolism	73.9 ± 8.55	61.6 ± 4.51	0.170
Glycolysis / Gluconeogenesis	98.5 ± 12.8	97.8 ± 7.56	0.948
Glyoxylate and dicarboxylate metabolism	26.0 ± 2.41	38.7 ± 3.04	0.009
Inositol phosphate metabolism	7.07 ± 0.79	6.71 ± 0.54	0.231
Pentose and glucuronate interconversions	37.2 ±3.31	45.6 ± 3.72	0.018
Pentose phosphate pathway	62.4 ± 7.80	75.8 ± 6.07	0.081
Propanoate metabolism	41.6 ± 4.65	41.4 ± 2.93	0.943
Pyruvate metabolism	76.0 ± 9.28	90.3 ± 6.66	0.016
Starch and sucrose metabolism	78.4 ± 11.8	84.8 ± 7.13	0.467
Amino acid metabolism			
Alanine, aspartate, and glutamate metabolism	53.5 ± 6.54	81.4 ± 6.34	0.002
Amino acid related enzymes	89.0 ± 11.1	117 ± 8.77	0.005
Arginine and proline metabolism	60.4 ± 6.63	96.2 ± 7.53	0.006
Cysteine and methionine metabolism	55.9 ± 6.94	75.3 ± 5.45	0.005
Glycine, serine, and threonine metabolism	46.5 ± 5.67	61.3 ± 4.46	0.002
Histidine metabolism	27.6 ± 3.62	50.3 ± 3.82	0.022
Lysine biosynthesis	41.8 ± 5.51	68.4 ± 5.36	0.002
Lysine degradation	7.50 ± 0.80	8.00 ± 0.57	0.189
Phenylalanine metabolism	7.19 ± 0.95	13.8 ± 1.19	0.002
Phenylalanine, tyrosine, and tryptophan biosynthesis	33.3 ± 4.87	68.4 ± 5.83	0.002
Tryptophan metabolism	12.6 ± 1.55	8.55 ± 0.62	0.033
Tyrosine metabolism	30.9 ± 3.63	28.3 ± 2.03	0.389
Valine, leucine, and isoleucine biosynthesis	47.1 ± 7.72	63.3 ± 5.08	0.009
Valine, leucine, and isoleucine degradation	18.4 ± 2.23	12.2 ± 0.75	0.025
Metabolism of other amino acids			
Cyanoamino acid metabolism	19.0 ± 3.32	18.5 ± 1.61	0.859
D-Alanine metabolism	11.6 ± 1.66	8.76 ± 0.60	0.088

D-Arginine and D-ornithine metabolism	0.11 ± 0.05	0.14 ± 0.02	0.560
D-Glutamine and D-glutamate metabolism	10.0 ± 1.24	11.6 ± 0.81	0.142
Glutathione metabolism	18.9 ± 2.66	11.4 ± 0.79	0.026
Phosphonate and phosphinate metabolism	5.38 ± 0.58	5.71 ± 0.76	0.687
Selenocompound metabolism	26.0 ± 3.13	26.9 ± 2.02	0.765
Taurine and hypotaurine metabolism	9.28 ± 1.12	7.30 ± 0.50	0.113
β-Alanine metabolism	6.29 ± 0.53	10.5 ± 1.00	0.015

[1] Values are means ± SEM; n = 5. The effect of GIT location was tested using a paired t test. GIT, gastrointestinal tract; KEGG, Kyoto Encyclopaedia of Genes and Genomes; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states.

Supplementary Table 5.3: SCFA production during *in vitro* ileal and hindgut fermentation of pigs fed a human-type diet[1]

SCFA	GIT location		*P* value
	Ileum	Hindgut	
	mmol/kg DM incubated		
Acetate	384 ± 65.7	121 ± 70.2	0.090
Propionate	11.5 ± 2.19	97.6 ± 19.2	0.022
Butyrate	6.90 ± 4.60	113 ± 13.0	0.011
Iso-butyrate	1.06 ± 0.17	4.82 ± 1.50	0.062
Iso-valerate	4.28 ± 0.73	6.25 ± 1.24	0.020
Valerate	1.71 ± 1.00	14.7 ± 1.40	0.002

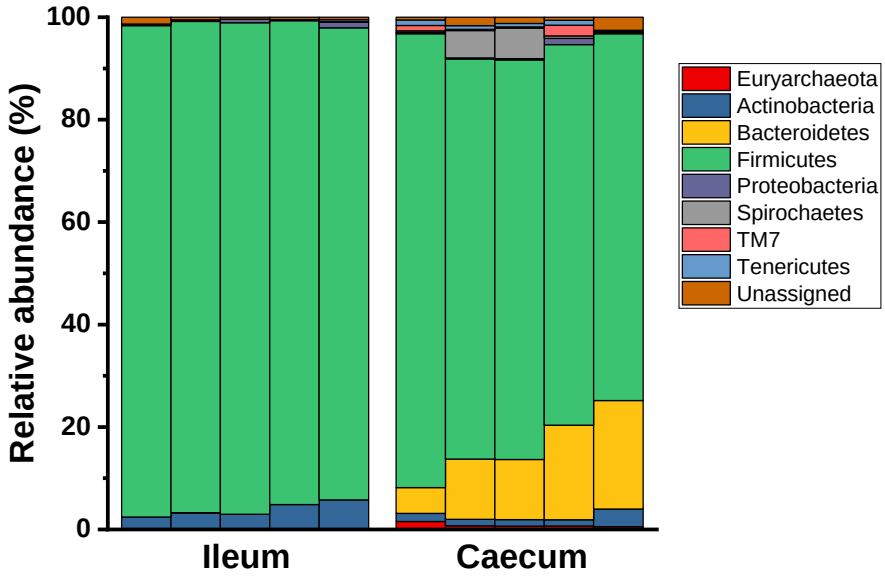
[1] Values are means ± SEM; *n* = 5. The effect of GIT location was tested using a paired *t* test. DM, dry matter; GIT, gastrointestinal tract; SCFA, short-chain fatty acid.

Supplementary Table 5.4: Nutrient composition and nutrient content (normalised) of terminal jejunum and terminal ileal digesta of pigs fed a human-type diet[1]

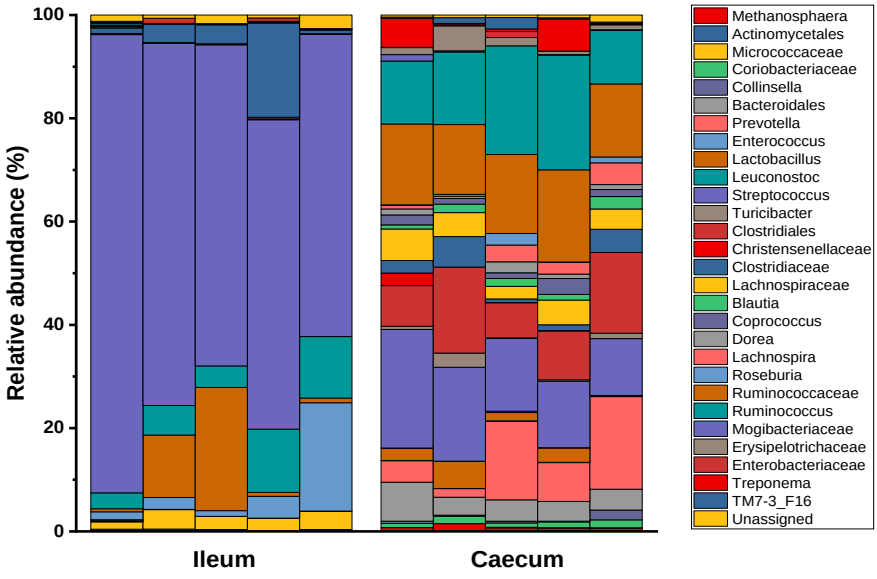
	GIT location		*P* value
	Terminal jejunum	Terminal ileum	
Nutrient composition, *g/kg DM*			
Organic matter	891 ± 76	834 ± 9.49	0.015
Crude protein	236 ± 8.68	188 ± 9.13	0.012
Lipid	12.4 ± 3.64	14.7 ± 2.38	0.466
Starch	107 ± 15.2	82.0 ± 6.27	0.214
Normalised nutrient content, *g/kg diet DM intake*			
Organic matter	246 ± 21.8	141 ± 10.3	0.010
Crude protein	65.4 ± 6.69	32.0 ± 2.90	0.004
Lipid	3.51 ± 1.12	2.53 ± 0.52	0.291
Starch	30.7 ± 6.5	13.9 ± 1.54	0.067

[1] Values are means ± SEM; *n* = 5. Terminal jejunum and terminal ileal digesta were the substrate for ileal and hindgut fermentation, respectively. The effect of GIT location was tested using a paired t test. DM, dry matter; GIT, gastrointestinal tract.

Supplementary figures



Supplementary Figure 5.1: Relative abundance of archaeal and bacterial phyla in ileal and caecal digesta of pigs fed a human-type diet (*n* = 5).



Supplementary Figure 5.2: Relative abundance of archaeal and bacterial genera in ileal and caecal digesta of pigs fed a human-type diet (*n* = 5).

Appendix 4

Chapter 6: Supplementary material

Supplemental methods

Microbial analysis

Quantitative PCR. The total bacteria concentration was determined using a LightCycler 480 Real-Time PCR instrument (Roche) as described previously [286]. In short, *Escherichia coli* (Nissle) was used to prepare a standard curve. DNA was amplified using SyBr Green detection chemistry (Roche). Total reaction volume included 10 µL Master mix, 2 µL forward-primer (5'-TCCTACGGGAGGCAGCAGT) (5 µM), 2 µL reverse-primer (5'-GGACTACCAGGGTATCTAATCCTGTT) (5 µM), 1 µL water, and 5 µL extracted DNA.

16S rRNA gene sequencing and bioinformatics. The Illumina MiSeq sequencing was performed at the Massey Genome Service (Massey University) [286]. Primers were used to amplify the V3-V4 hypervariable region of the 16S rRNA gene, 16SF_V3 (5' - AATGATACGGCGACCACCGAGATCTACAC-index-TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). The sequencing data were analysed using Mothur V1.44.1 [334, 378]. Briefly, 12,340,891 paired-end reads were assembled before undergoing quality control; reads with more than eight homopolymers and uncalled bases were removed. The average length of the sequences was 420 bp. The sequences were aligned against the SLIVA database (version 138) [379] and the Greengenes database (version 13_8) [338]. After alignment and filtering, a pre-clustering step (4 bp) was performed to denoise and reduce the effect of sequencing errors. Chimeras were removed using VSEARCH [380]. The non-bacterial sequences were removed. The remaining 8,136,634

reads were clustered into OTUs with a 97% cut off. A subsample of 100,567 reads per sample was. The BIOM tables were exported and uploaded in Calypso (version 8.84) [381] and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [382]. Calypso was used to obtain the taxonomic composition and Shannon diversity numbers and perform principal coordinates analysis (PCoA) according to Bray-Curtis dissimilarities. PICRUSt was used to predict the metabolic activity of the ileal and faecal microbial communities.

Calculations

To determine *in vitro* OM fermentation for either ileal or hindgut fermentation, the following equations were used as described previously [292]:

1) $\text{OM fermentation}_{\textit{in vitro}}\,(\%) = (\text{OM}_{\text{before } \textit{in vitro} \text{ fermentation}} - [\text{OM}_{\text{after } \textit{in vitro} \text{ fermentation}} - ((\text{OM}_{\text{blank initial}} + \text{OM}_{\text{blank final}})/2)]) / \text{OM}_{\text{before } \textit{in vitro} \text{ fermentation}} \times 100$

where $\text{OM}_{\text{blank initial}}$ and $\text{OM}_{\text{blank final}}$ are the amounts of OM in the blanks prior to (initial) and after (final) *in vitro* fermentation, respectively. For each diet, the *in vitro* ileal and hindgut fermentation had their own blanks.

The calculation used to determine the normalised nutrient contents was as follows:

2) $\text{Normalised nutrient content}_{\text{terminal jejunal or terminal ileal digesta}}\,(\text{g/kg diet DM intake}) = \text{nutrient concentration}_{\text{terminal jejunal or terminal ileal digesta}}\,(\text{g/kg DM}) \times (\text{TiO}_{2\text{-diet}}/\text{TiO}_{2\text{-terminal jejunum or terminal ileal digesta}})$

where $\text{TiO}_{2\text{-diet}}$ and $\text{TiO}_{2\text{-terminal jejunal or terminal ileal digesta}}$ are the TiO_2 (g/kg DM) in the diet and digesta, respectively.

The *in vivo* ileal and hindgut OM fermentation were determined as follows:

3) OM fermentation$_{in\ vivo}$ (%) = ((Normalised OM content$_{\text{terminal jejunal or terminal ileal digesta}}$ (g/kg DM) – Normalised OM content $_{\text{terminal ileal digesta or faeces}}$) / Normalised OM content$_{\text{terminal jejunal or terminal ileal digesta}}$) x 100

The estimated *in vivo* / *in vitro* ileal and hindgut production of SCFAs was determined as described previously [292] using the following equations:

4) Production of SCFA$_{\text{ileal or hindgut}}$ *in vitro* (mmol/kg substrate DM incubated) = (SCFA$_{\text{after}}$ *in vitro* fermentation (mmol/kg DM) – [(SCFA$_{\text{blank initial}}$ + SCFA$_{\text{blank final}}$)/2])
5) Estimated production of SCFA$_{\text{ileal or hindgut}}$ *in vivo* / *in vitro* (mmol/kg diet DM intake) = production of SCFA$_{\text{ileal or hindgut}}$ *in vitro* (mmol/kg substrate DM incubated) x ($TiO_{2\text{-diet}}$/$TiO_{2\text{-terminal jejunal or terminal ileal digesta}}$)

where SCFAs$_{\text{blank initial}}$ and SCFAs$_{\text{blank final}}$ are the SCFAs (mmol/kg DM) in the blanks prior to (initial) and after (final) *in vitro* fermentation, respectively. For each diet, the *in vitro* ileal and hindgut fermentation had their own blanks.

The normalised total number of bacteria and the number per taxa in ileal digesta and faeces were calculated as follows [286]:

6) Normalised total number of bacteria$_{\text{ileal digesta or faeces}}$ (16S rRNA gene copy number/kg DM intake) = number of bacteria$_{\text{ileal digesta or faeces}}$ (16S rRNA gene copy number/kg DM) x ($TiO_{2\text{-diet}}$/ $TiO_{2\text{-terminal ileal digesta or faeces}}$)
7) Normalised number of bacteria per taxa$_{\text{ileal digesta or faeces}}$ (16S rRNA gene copy number/kg DM intake) = normalised total number of bacteria$_{\text{ileal digesta or faeces}}$ (16S rRNA gene copy number/kg DM intake) x relative abundance$_{\text{phylum/genus}}$ (%) / 100

Supplementary tables

Supplementary Table 6.1: Ingredient and determined nutrient composition of CEL, KF, and PSY diets[1]

	CEL	CEL+KF	CEL+PSY
Ingredient, g/kg DM			
CEL[2]	45	23	23
PSY[2]	-	-	24
KF (DM)[2,3]	-	110	-
Wheat starch	522	482	519
Lactic casein	210	205	210
Sucrose	141	102	141
Soya oil	50	46	50
Dicalcium phosphate	20	20	20
Sodium chloride	3	3	3
Calcium carbonate	1	1	1
Vitamin/mineral mix[4]	5	5	5
Titanium dioxide	3	3	3
Nutrient, g/kg DM			
Ash	35	39	36
Crude protein	175	198	178
Total fat	48	51	49
Total starch	405	431	417
Total sugars	121	132	123
Total dietary fibre	45	44	45
Insoluble dietary fibre[5]	45	33	24
Soluble dietary fibre[5]	0	11	21
Titanium dioxide	3	3	3
Gross energy, MJ/kg	17.4	18.2	17.4

[1] CEL, cellulose; DM, dry matter, KF, kiwifruit; PSY, psyllium.
[2] The total dietary fibre content was 100, 91, and 19% for CEL, PSY, and KF pulp, respectively.
[3] Freshly peeled and crushed KF was added to the diet immediately before feeding.
[4] Vitamin and mineral premixes were obtained from Vitec Nutrition Ltd (Auckland, New Zealand) and supplied (per kg of diet as-fed): Mn, 45 mg; Zn, 80 mg; Cu, 25 mg; Co, 0.5 mg; Se, 0.3 mg; Fe, 100 mg; I, 1.0 mg; choline, 100 mg; all-trans retinyl acetate, 3.0 mg; cholecalciferol, 0.05 mg; α-tocopherol, 50 mg; menadione, 2.0 mg; thiamine, 1.0 mg; riboflavin, 3.0 mg; nicotinic acid, 15 mg; pantothenic acid, 20 mg; pyridoxine, 2.0 mg; cyanocobalamin, 0.01 mg; folic acid, 0.5 mg; biotin, 0.1 mg.
[5] Calculated values based on reported percentages of soluble dietary fibre in PSY (85%) [383] and KF (47%) [354].

Supplementary Table 6.2: Frequency of occurrence of taxa in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for forty-two days[1]

Phylum	Genus	Ileal digesta				*P* value[2]	Faeces				*P* value[2]	*P* value
		CEL	CEL +KF	CEL +PSY	SEM	Diet	CEL	CEL +KF	CEL +PSY	SEM	Diet	GIT region
Sample size, *n*[3]		7	7	5			7	8	6			
Actinobacteriota		1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
	Bifidobacterium	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
	Collinsella	0.143	0.714	0.600	0.178	0.159	1.00*	1.00	1.00	0.000	-	<0.001
	Eggerthellaceae[4]	0.286	0.286	0.600	0.188	0.494	1.00*	1.00*	1.00	0.000	-	<0.001
	Enorma	0.143^{ab}	0.571^{a}	0.000^{b}	0.132	0.004	1.00*	1.00*	1.00*	0.000	-	0.028
	Olsenella	0.571^{b}	1.00^{a}	0.600^{ab}	0.166	0.029	1.00*	1.00	1.00	0.000	-	0.029
Bacteroidota		1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
	Alloprevotella	0.857	0.857	1.00	0.108	0.288	1.00	1.00	1.00	0.000	-	0.288
	Bacteroidales[4]	0.857	1.00	1.00	0.076	0.288	1.00	1.00	1.00	0.000	-	0.288
	Bacteroides	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
	Bacteroidia[4]	0.143	0.286	0.000	0.125	0.103	1.00*	1.00*	1.00*	0.000	-	<0.001
	Muribaculaceae[4]	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
	Parabacteroides	0.571^{b}	1.00^{a}	0.600^{ab}	0.166	0.029	1.00*	1.00	1.00	0.000	-	0.029
	Prevotellaceae[4]	0.571^{b}	1.00^{a}	0.600^{ab}	0.166	0.029	1.00*	1.00	1.00	0.000	-	0.029
	Prevotella	1.00	0.857	1.00	0.076	0.288	1.00	1.00	1.00	0.000	-	0.288
	Prevotellaceae_NK3B31_group	0.714	0.857	0.400	0.178	0.459	1.00	1.00	1.00*	0.000	-	0.010
	Prevotellaceae_UCG001	0.143	0.000	0.400	0.148	0.077	1.00*	0.875*	1.00*	0.067	0.775	0.010
	Prevotellaceae_UCG003	0.000	0.286	0.000	0.099	0.103	1.00*	1.00*	1.00*	0.000	-	<0.001
	Rikenellaceae_RC9_gut_group	0.000^{b}	1.00^{a}	0.400^{b}	0.126	0.010	1.00*	1.00	1.00*	0.000	-	0.010

Tannerellaceae[4]	0.143	0.429	0.400	0.183	0.508	1.00*	1.00*	1.00*	0.000	-	0.010
Desulfobacterota	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Fibrobacterota	0.143	0.286	0.000	0.125	0.103	1.00*	0.875*	1.00*	0.067	0.293	<0.001
Fibrobacter	0.000	0.143	0.000	0.076	0.288	1.00*	0.875*	1.00*	0.067	0.293	<0.001
Firmicutes	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Acidaminococcus	0.286	0.286	0.400	0.188	0.896	1.00*	0.750*	0.00*	0.088	0.112	0.002
Anaerovoracaceae[4]	0.714	0.714	0.800	0.173	0.934	1.00	1.00	1.00	0.000	-	0.103
Blautia	1.00	0.857	1.00	0.076	0.288	1.00	1.00	1.00	0.000	-	0.288
Butyricicoccaceae_UCG008	0.000	0.000	0.000	0.000	-	0.429[b]*	1.00[a]*	1.00[a]*	0.108	0.001	0.033
Carnobacterium	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Catenisphaera	0.429	0.286	0.200	0.179	0.700	1.00*	1.00*	1.00*	0.000	-	0.004
Cellulosilyticum	0.143	0.429	0.400	0.183	0.485	0.143	0.625	0.333	0.167	0.217	0.698
Christensenellaceae_R7_group	0.857	0.875	0.800	0.149	0.799	1.00	1.00	1.00	0.000	-	0.271
Clostridium_sensu_stricto_1	1.00	1.00	1.00	0.000	-	0.857	1.00	1.00	0.076	0.293	0.293
Clostridia_UCG014[4]	0.571	0.857	0.400	0.183	0.319	1.00*	1.00	1.00*	0.000	-	0.029
Colidextribacter	0.429	0.286	0.600	0.193	0.577	1.00*	1.00*	1.00	0.000	-	0.004
Coprococcus	0.143[ab]	0.571[a]	0.000[b]	0.132	0.004	1.00*	1.00*	0.833*	0.088	0.288	0.028
Dorea	0.714	0.571	0.600	0.193	0.946	1.00	1.00*	1.00	0.000	-	0.029
Enterococcus	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Erysipelotrichaceae[4]	0.714	0.571	0.600	0.193	0.848	1.00	1.00*	1.00	0.000	-	0.029
Erysipelotrichaceae_UCG006	0.000	0.000	0.000	0.000	-	0.857[ab]*	0.500[b]*	1.00[a]*	0.127	0.008	0.010
Erysipelotrichaceae_UCG009	0.286[a]	0.000[b]	0.200[a]	0.143	0.047	0.857[a]*	0.375[b]*	1.00[a]*	0.125	0.047	0.004
Family_XIII_AD3011_group	0.571[b]	1.00[a]	0.400[b]	0.166	0.029	1.00*	1.00	1.00*	0.000	-	0.029
Holdemanella	0.000	0.143	0.200	0.128	0.271	1.00*	0.875*	1.00*	0.067	0.293	<0.001
Lachnoclostridium	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Lachnospira	0.286	0.286	0.200	0.173	0.934	0.857*	1.00*	0.667*	0.135	0.099	0.002
Lachnospiraceae[4]	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-

Lachnospiraceae_NK3A20_group	0.429[a]	0.429[a]	0.000[b]	0.153	0.028	1.00[a]*	0.625[b]	1.00[a]*	0.098	0.034	0.003
Lachnospiraceae_NK4A136_group	0.286	0.571	0.600	0.193	0.485	1.00*	1.00*	1.00	0.000	-	0.029
Lactobacillus	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Lactococcus	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Megamonas	0.000	0.143	0.000	0.076	0.288	0.000	0.250	0.333	0.142	0.103	0.225
Megasphaera	0.286	0.429	0.400	0.193	0.848	1.00*	0.750	1.00*	0.089	0.112	0.003
Mitsuokella	0.143	0.000	0.200	0.128	0.271	1.00[a]*	0.250[b]*	0.833[b]*	0.125	0.014	0.003
Oscillospirales[4]	0.857	1.00	0.600	0.148	0.627	1.00	1.00	1.00	0.000	-	0.077
Oscillospiraceae[4]	0.429	0.857	0.600	0.183	0.317	1.00*	1.00	1.00	0.000	-	0.004
Oscillospiraceae_NK4A214_group	0.000[b]	0.571[a]	0.200[ab]	0.149	0.004	1.00*	1.00*	1.00*	0.000	-	0.029
Oscillospiraceae_UCG002	0.714	1.00	1.00	0.099	0.104	1.00	1.00	1.00	0.000	-	0.093
Oscillospiraceae_UCG005	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Phascolarctobacterium	0.571	0.571	0.800	0.184	0.383	1.00*	1.00*	1.00	0.000	-	0.028
Romboutsia	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Ruminococcaceae[4]	0.714	0.714	0.600	0.188	0.896	1.00	1.00	1.00	0.000	-	0.103
Ruminococcaceae_UBA1819	0.143	0.143	0.200	0.149	0.962	1.00*	1.00*	1.00*	0.000	-	<0.001
Ruminococcus	0.714[ab]	1.00[a]	0.400[b]	0.159	0.010	1.00	1.00	1.00*	0.000	-	0.010
Selenomonadaceae[4]	0.143	0.857	0.400	0.166	0.187	1.00*	0.875	1.00*	0.067	0.293	0.008
Selenomonas	0.571	0.857	0.400	0.183	0.721	1.00	0.750	0.833	0.183	0.118	0.110
Solobacterium	0.429	0.571	0.400	0.198	0.809	1.00*	1.00*	0.833*	0.088	0.286	0.008
Streptococcus	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Subdoligranulum	0.714	0.714	0.600	0.188	0.683	1.00	1.00	1.00	0.000	-	0.103
Succiniclasticum	0.429	0.286	0.000	0.146	0.858	0.429	0.625	0.667	0.184	0.651	0.060
Syntrophococcus	0.429	0.429	0.400	0.198	0.994	1.00*	0.875*	1.00*	0.068	0.293	0.006
Terrisporobacter	0.143[b]	0.571[a]	0.400[a]	0.183	0.021	0.143[b]	0.875[a]	0.667[a]	0.151	0.021	0.186
Weissella	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Fusobacteriota	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-

Fusobacterium	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Patescibacteria	1.00	0.714	1.00	0.099	0.104	0.714*	0.500*	0.500*	0.184	0.660	0.038
Candidatus_Saccharimonas	0.286	0.143	0.200	0.162	0.812	0.571	0.250	0.333	0.178	0.456	0.226
Proteobacteria	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Anaerobiospirillum	0.143	0.286	0.000	0.125	0.105	1.00*	0.750*	1.00*	0.087	0.107	<0.001
Budvicia	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Citrobacter	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Cronobacter	0.857	0.857	1.00	0.108	0.288	0.143*	0.250*	0.500*	0.166	0.399	0.001
Desulfovibrio	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Enterobacterales[4]	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Escherichia-Shigella	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Klebsiella	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Serratia	1.00	1.00	1.00	0.000	-	1.00	1.00	1.00	0.000	-	-
Succinivibrionaceae[4]	0.000	0.286	0.200	0.143	0.103	0.857^{ab}*	0.375^{b}*	1.00^{a}*	0.125	0.038	0.003
Succinivibrio	0.143	0.429	0.400	0.183	0.508	1.00*	1.00*	1.00*	0.000	-	0.010
Spirochaetota	0.286^{b}	1.00^{a}	0.400^{ab}	0.159	0.010	1.00*	1.00*	1.00*	0.000	-	0.010
Treponema	0.286^{b}	1.00^{a}	0.400^{b}	0.159	0.010	1.00*	1.00	1.00*	0.000	-	0.010
Synergistota	0.286	0.571	0.600	0.193	0.485	1.00*	1.00*	1.00	0.000	-	0.028
Cloacibacillus	0.000	0.143	0.000	0.076	0.288	1.00*	1.00*	1.00*	0.000	-	<0.001
Pyramidobacter	0.000	0.143	0.200	0.128	0.271	0.714*	0.750*	0.833*	0.132	0.880	0.001
Synergistaceae[4]	0.000	0.143	0.000	0.077	0.288	0.857*	0.875*	0.833*	0.111	0.976	<0.001

[1] Values are frequencies. Only taxa with >1% abundance were included. The frequency analysis was performed using a binary logistic regression for each GIT region with 0 when the taxon was not present and 1 when the taxon was present with a relative abundance >0.1%. The means within each GIT region with a different letter differ ($P \leq 0.05$), and the means with an asterisk for the faeces differ ($P \leq 0.05$) from their ileal digesta counterparts. CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; PSY, psyllium.

[2] If the taxon was present in all samples of one (or both) GIT region(s) (i.e., frequency equals 1.00), no statistical analysis was conducted for that GIT region. Likewise, if the taxon was present in none of the samples of one GIT region (i.e., frequency equals 0.000), no statistical analysis was conducted for that GIT region. The diet effect was determined for each GIT region individually.

[3] n indicates the number of replicates. The different numbers of replicates are due to insufficient digesta or faeces collected for analysis.

[4] Bacteria could only be classified as far as order or family level.

Supplementary Table 6.3: Taxonomic composition in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for forty-two days[1]

Phylum	Genus	Ileal digesta				Faeces				*P* value[2]		
		CEL	CEL +KF	CEL +PSY	SEM	CEL	CEL +KF	CEL +PSY	SEM	Diet	GIT region	Interaction
Sample size, *n*[3]		7	7	5		7	8	6				
		log$_{10}$ 16S rRNA gene copies/kg diet DM intake										
Total bacteria		13.2^{b}	13.1^{b}	13.6^{a}	0.177	12.8^{b}	12.8^{b}	13.3^{a}	0.112	0.007	0.057	-
Actinobacteriota		10.7	10.5	10.7	0.194	11.6*	11.4*	11.6*	0.138	0.575	<0.001	-
	Bifidobacterium	9.48	9.50	9.38	0.261	9.18	9.21	9.08	0.255	0.920	0.251	-
	Collinsella	ND	ND	ND	-	10.3^{b}	10.1^{b}	11.2^{a}	0.225	0.003	-	-
	Eggerthellaceae[4]	7.63	7.64	7.86	0.238	10.1*	10.1*	10.4*	0.159	0.220	<0.001	-
	Enorma	ND	ND	ND	-	9.83	10.6	9.18	0.367	0.052	-	-
	Olsenella	8.63	8.28	8.58	0.322	10.9*	10.6*	10.9*	0.314	0.608	<0.001	-
Bacteroidota		10.9^{b}	10.9^{b}	11.3^{a}	0.157	12.3^{b}*	12.3^{b}*	12.7^{a}*	0.117	0.019	<0.001	-
	Alloprevotella	8.84	8.84	9.40	0.307	10.1*	10.1*	10.7*	0.226	0.139	<0.001	-
	Bacteroidales[4]	8.61^{b}	9.07^{ab}	9.49^{a}	0.234	10.3^{b}*	10.7^{ab}*	11.1^{a}*	0.177	0.044	<0.001	-
	Bacteroides	10.7	10.6	11.0	0.168	11.9*	11.8*	12.3*	0.163	0.086	<0.001	-
	Bacteroidia[4]	ND	ND	ND	-	9.34^{b}	9.71^{ab}	10.4^{a}	0.306	0.013	-	-
	Muribaculaceae[4]	9.64	9.55	9.84	0.197	10.6*	10.6*	10.8*	0.193	0.505	<0.001	-
	Parabacteroides	8.52^{b}	8.95^{ab}	9.15^{a}	0.271	10.8^{b}*	11.3^{ab}*	11.5^{a}*	0.168	0.037	<0.001	-
	Prevotellaceae[4]	8.66	8.50	8.76	0.282	11.3*	11.1*	11.4*	0.195	0.607	<0.001	-
	Prevotella	9.38^{ab}	8.62^{b}	9.72^{a}	0.291	11.0^{ab}*	10.2^{b}*	11.3^{a}*	0.286	0.045	<0.001	-
	Prevotellaceae NK3B31 group	8.09^{b}	8.51^{ab}	9.05^{a}	0.318	10.4^{b}*	10.8^{ab}*	11.4^{a}*	0.218	0.015	<0.001	-
	Prevotellaceae UCG001	ND	ND	ND	-	9.27^{ab}	8.96^{b}	10.6^{a}	0.371	0.013	-	-
	Prevotellaceae UCG003	ND	ND	ND	-	9.88	9.95	9.87	0.307	0.979	-	-
	Rikenellaceae RC9 gut group	ND	ND	ND	-	10.2	10.7	10.7	0.269	0.197	-	-

Tannerellaceae[4]	ND	ND	ND	-	9.97[b]	10.5[ab]	11.1[a]	0.242	0.022	-	-
Desulfobacterota	9.41[ab]	9.20[b]	9.82[a]	0.202	11.2[ab]*	11.0[b]*	11.6[a]*	0.115	0.003	<0.001	-
Fibrobacterota	ND	ND	ND	-	9.18	9.95	10.5	0.578	0.294	-	-
Fibrobacter	ND	ND	ND	-	9.18	9.94	10.5	0.578	0.297	-	-
Firmicutes	12.8[b]	12.9[ab]	13.1[a]	0.144	12.5[b]*	12.5[ab]*	12.8[a]*	0.101	0.042	0.010	-
Acidaminococcus	7.76[ab]	6.66[b]	8.00[a]	0.345	10.6[ab]*	9.5[b]*	10.9[a]*	0.338	0.036	<0.001	-
Anaerovoracaceae[4]	8.82[a]	7.79[b]	8.57[ab]	0.284	10.1[a]*	9.11[b]*	9.89[ab]*	0.277	0.009	<0.001	-
Blautia	9.22	9.16	9.71	0.284	10.5*	10.4*	11.0*	0.217	0.159	<0.001	-
Butyricicoccaceae UCG008	ND	ND	ND	-	8.28	9.60	9.12	0.549	0.232	-	-
Carnobacterium	11.5[b]	11.6[ab]	11.9[a]	0.152	10.4[b]*	10.5[ab]*	10.8[a]*	0.092	0.045	<0.001	-
Catenisphaera	ND	ND	ND	-	10.7[a]	9.01[b]	10.1[a]	0.212	<0.001	-	-
Christensenellaceae R7 group	8.49	8.73	8.92	0.249	11.0*	11.3*	11.5*	0.242	0.370	<0.001	-
Clostridium sensu stricto 1	9.34[b]	11.2[a]	10.3[b]	0.310	8.91[b]	10.8[a]	9.84[b]	0.302	<0.001	0.170	-
Clostridia UCG014[4]	8.40	8.89	8.44	0.395	10.2*	10.7*	10.2*	0.385	0.498	<0.001	-
Colidextribacter	7.90	8.02	8.27	0.305	9.92*	10.0*	10.3*	0.297	0.633	<0.001	-
Coprococcus	ND	ND	ND	-	9.94	10.2	10.0	0.448	0.884	-	-
Dorea	8.02	8.35	8.68	0.316	10.0*	10.3*	10.7*	0.308	0.256	<0.001	-
Enterococcus	10.5[b]	10.7[ab]	11.1[a]	0.207	9.6[b]*	9.8[ab]*	10.2[a]*	0.124	0.008	<0.001	-
Erysipelotrichaceae[4]	8.06	8.11	8.80	0.328	9.82*	9.87*	10.6*	0.319	0.155	<0.001	-
Erysipelotrichaceae UCG006	ND	ND	ND	-	10.2[a]	7.95[b]	10.3[a]	0.484	0.003	-	-
Erysipelotrichaceae UCG009	ND	ND	ND	-	9.55[a]	7.64[b]	9.55[a]	0.474	0.011	-	-
Family XIII AD3011 group	7.87[b]	8.72[a]	8.81[a]	0.209	10.5[b]*	11.3[a]*	11.4[a]*	0.164	<0.001	<0.001	-
Holdemanella	ND	ND	ND	-	9.29[b]	9.45[ab]	10.2[a]	0.300	0.009	-	-
Lachnoclostridium	9.08	9.39	9.77	0.237	9.33	9.64	10.0	0.233	0.060	0.196	-
Lachnospira	ND	ND	ND	-	8.99	9.88	8.73	0.426	0.153	-	-
Lachnospiraceae[4]	10.1[ab]	9.75[b]	10.3[a]	0.181	11.8[ab]*	11.4[b]*	12.0[a]*	0.118	0.012	<0.001	-

Lachnospiraceae NK3A20 group	ND	ND	ND	-	10.2[a]	7.97[b]	10.5[a]	0.295	<0.001	-	-
Lachnospiraceae NK4A136 group	7.87	8.40	7.81	0.305	9.47*	10.0*	9.41*	0.297	0.209	<0.001	-
Lactobacillus	12.1[a]	11.4[b]	12.2[a]	0.197	10.5[a]*	9.80[b]*	10.7[a]*	0.158	<0.001	<0.001	-
Lactococcus	12.7	12.7	12.2	0.234	10.2*	10.8*	10.7*	0.219	0.448	<0.001	0.023
Megasphaera	7.81	7.62	8.04	0.447	10.6[a]*	8.81[b]	11.4[a]*	0.423	0.004	<0.001	0.039
Mitsuokella	ND	ND	ND	-	10.1[a]	7.49[b]	8.94[b]	0.373	<0.001	-	-
Oscillospirales[4]	8.55[b]	8.94[ab]	8.94[a]	0.192	11.3[b]*	11.6[ab]*	11.6[a]*	0.146	0.016	<0.001	-
Oscillospiraceae[4]	7.90	8.24	8.46	0.243	10.2*	10.5*	10.8*	0.237	0.183	<0.001	-
Oscillospiraceae NK4A214 group	ND	ND	ND	-	10.8	10.8	11.1	0.167	0.068	-	-
Oscillospiraceae UCG002	8.84[b]	8.86[ab]	9.32[a]	0.233	10.6[b]*	10.9[ab]*	11.4[a]*	0.228	0.007	<0.001	-
Oscillospiraceae UCG005	9.08	9.35	9.44	0.225	10.7*	11.0*	11.1*	0.221	0.260	<0.001	-
Phascolarctobacterium	8.10[b]	8.24[ab]	8.79[a]	0.268	10.8[b]*	10.9[ab]*	11.5[a]*	0.166	0.018	<0.001	-
Romboutsia	9.71[b]	10.8[a]	10.1[ab]	0.259	8.98[b]*	10.1[a]*	9.37[ab]*	0.252	0.0.03	0.007	-
Ruminococcaceae[4]	8.26	8.50	8.68	0.284	10.1*	10.3*	10.5*	0.276	0.489	<0.001	-
Ruminococcaceae UBA1819	ND	ND	ND	-	9.98	9.82	10.5	0.360	0.451	-	-
Ruminococcus	8.29	8.72	8.28	0.326	10.5*	10.9*	10.4*	0.317	0.419	<0.001	-
Selenomonadaceae[4]	ND	ND	ND	-	10.8[a]	9.23[b]	10.2[ab]	0.385	0.027	-	-
Selenomonas	8.69	8.04	8.40	0.400	9.72*	9.07*	9.43*	0.390	0.375	0.012	-
Solobacterium	8.15	7.93	7.68	0.352	10.2*	9.96*	9.71*	0.343	0.568	<0.001	-
Streptococcus	11.3	11.2	11.6	0.135	10.0*	9.93*	10.3*	0.093	0.081	<0.001	-
Subdoligranulum	8.60	8.21	8.96	0.297	10.9*	10.6*	11.3*	0.210	0.090	<0.001	-
Succiniclasticum	ND	ND	ND	-	8.14	7.74	9.18	0.619	0.275	-	-
Syntrophococcus	8.06	7.26	7.90	0.272	10.6*	9.81*	10.4*	0.224	0.100	<0.001	-
Weissella	11.6	11.9	11.3	0.235	8.58[b]*	9.48[a]*	9.62[a]*	0.219	0.019	<0.001	0.004
Fusobacteriota	9.76	9.88	10.4	0.261	10.5*	10.6*	11.1*	0.254	0.112	0.010	-
Fusobacterium	9.69	9.84	10.4	0.257	10.43	10.6*	11.1*	0.250	0.087	0.006	-
Patescibacteria	9.13	8.61	8.95	0.327	8.15*	7.62*	7.97*	0.320	0.509	0.002	-

Candidatus Saccharimonas	ND	ND	ND	-	7.83	7.45	7.67	0.470	0.838	-	-
Proteobacteria	12.8^{b}	12.4^{c}	13.4^{a}	0.188	11.7^{b}*	11.3^{c}	12.3^{a}*	0.117	<0.001	<0.001	-
Anaerobiospirillum	ND	ND	ND	-	10.1^{a}	8.83^{b}	10.2^{a}	0.418	0.049	-	-
Budvicia	10.8	10.7	11.0	0.121	9.72*	9.71*	9.93*	0.092	0.150	<0.001	-
Citrobacter	10.7	10.8	11.0	0.128	9.65*	9.76*	9.90*	0.111	0.257	<0.001	-
Cronobacter	10.1	8.49	9.23	0.523	-	-	-	-	0.201	-	-
Desulfovibrio	9.37^{ab}	9.14^{b}	9.76^{a}	0.202	11.2^{ab}*	10.9^{b}*	11.5^{a}*	0.115	0.003	<0.001	-
Enterobacterales[4]	10.8^{b}	10.5^{b}	11.4^{a}	0.189	9.54^{b}*	9.24^{b}*	10.1^{a}*	0.117	<0.001	<0.001	-
Escherichia-Shigella	12.7^{a}	11.4^{b}	13.3^{a}	0.208	11.2^{b}*	11.0^{b}	12.1^{a}*	0.197	<0.001	<0.001	0.013
Klebsiella	11.2^{ab}	10.5^{b}	12.2^{a}	0.374	9.92^{ab}*	9.15^{b}*	10.8^{a}*	0.296	0.002	<0.001	-
Serratia	11.4	11.4	11.6	0.122	10.3*	10.4*	10.5*	0.099	0.338	<0.001	-
Succinivibrionaceae[4]	ND	ND	ND	-	9.30^{a}	7.51^{b}	9.36^{a}	0.399	0.004	-	-
Succinivibrio	ND	ND	ND	-	10.5^{ab}	9.58^{b}	11.1^{a}	0.300	0.005	-	-
Spirochaetota	7.56^{b}	8.61^{a}	8.39^{ab}	0.312	10.2^{b}*	11.2^{a}*	11.0^{ab}*	0.304	0.016	<0.001	-
Treponema	7.55^{b}	8.61^{a}	8.38^{ab}	0.311	10.2^{b}*	11.2^{a}*	11.0^{ab}*	0.303	0.016	<0.001	-
Synergistota	7.68^{b}	8.12^{b}	9.19^{a}	0.310	9.88^{b}*	10.3^{b}*	11.4^{a}*	0.215	<0.001	<0.001	-
Cloacibacillus	ND	ND	ND	-	9.24^{b}	9.77^{b}	10.7^{a}	0.248	0.003	-	-
Pyramidobacter	ND	ND	ND	-	8.56	8.99	9.39	0.545	0.586	-	-
Synergistaceae[4]	ND	ND	ND	-	8.86	9.35	10.3	0.532	0.220	-	-

[1] Values are means with pooled SEM per GIT region. Only taxa with >1% relative abundance in at least one of the samples and with >25% frequency in the ileal digesta or faecal samples (Supplemental Table 2). Data were $\log_{10}$ transformed to achieve homogenous variance. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. Means within each GIT region with a different letter differ ($P \leq 0.05$), and the means with an asterisk for the faeces differ ($P \leq 0.05$) from their ileal digesta counterparts. CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; ND, not detected; PSY, psyllium.

[2] When the interaction between the diet and GIT region was not significant ($P > 0.05$), this interaction was removed from the model.

[3] *n* indicates the number of replicates. The different numbers of replicates are due to insufficient digesta or faeces collected for analysis.

[4] Bacteria could only be classified as far as order or family level.

Supplementary Table 6.4: Predicted metabolic activity (according to PICRUSt) related to fermentation (i.e., carbohydrate and protein metabolism) in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for 42 days[1]

KEGG reference pathway	Ileal digesta				Faeces				*P* value[2]		
	CEL	CEL +KF	CEL +PSY	SEM	CEL	CEL +KF	CEL +PSY	SEM	Diet	GIT region	Interaction
Sample size, *n*[3]	7	7	5		7	8	6				
	log₁₀ relative activity/kg diet DM intake										
Amino Acid Metabolism	11.1^{b}	10.9^{c}	10.3^{a}	0.039	10.2^{b*}	9.98^{c*}	10.4^{a*}	0.032	<0.001	<0.001	-
Alanine, aspartate, and glutamate metabolism	10.1^{b}	9.90^{c}	10.3	0.039	9.28^{b*}	9.02^{c*}	9.43^{a*}	0.033	<0.001	<0.001	-
Amino acid related enzymes	10.3^{a}	10.1^{b}	10.5^{a}	0.038	9.41^{a*}	9.18^{b*}	9.55^{a*}	0.031	<0.001	<0.001	-
Arginine and proline metabolism	10.1^{b}	9.93^{c}	10.3^{a}	0.039	9.29^{b*}	9.06^{c*}	9.46^{a*}	0.035	<0.001	<0.001	-
Cysteine and methionine metabolism	10.2^{b}	9.92^{c}	10.4^{a}	0.038	9.21^{b*}	8.96^{c*}	9.40^{a*}	0.032	<0.001	<0.001	-
Glycine, serine, and threonine metabolism	10.1^{b}	9.83^{c}	10.3^{a}	0.039	9.16^{b*}	8.92^{c*}	9.37^{a*}	0.032	<0.001	<0.001	-
Histidine metabolism	9.86^{a}	9.63^{b}	9.94^{a}	0.040	9.02^{a*}	8.79^{b*}	9.10^{a*}	0.035	<0.001	<0.001	-
Lysine biosynthesis	10.0^{a}	9.78^{b}	10.1^{a}	0.038	9.13^{a*}	8.89^{b*}	9.25^{a*}	0.032	<0.001	<0.001	-
Lysine degradation	9.55^{b}	9.14^{c}	9.83^{a}	0.056	8.35^{a*}	8.18^{b*}	8.56^{a*}	0.063	<0.001	<0.001	0.025
Phenylalanine metabolism	9.37^{b}	9.14^{c}	9.64^{a}	0.044	8.52^{b*}	8.29^{c*}	8.79^{a*}	0.037	<0.001	<0.001	-
Phenylalanine, tyrosine, and tryptophan biosynthesis	9.98^{b}	9.74^{c}	10.2^{a}	0.038	9.16^{b*}	8.92^{c*}	9.34^{a*}	0.032	<0.001	<0.001	-
Tryptophan metabolism	9.56^{b}	9.35^{c}	9.86^{a}	0.045	8.43^{b*}	8.23^{c*}	8.73^{a*}	0.040	<0.001	<0.001	-
Tyrosine metabolism	9.81^{a}	9.57^{b}	9.90^{a}	0.042	8.77^{a*}	8.53^{b*}	8.86^{a*}	0.033	<0.001	<0.001	-

Valine, leucine, and isoleucine biosynthesis	10.0[b]	9.79[c]	10.2[a]	0.039	9.07[b]*	8.85[c]*	9.26[a]*	0.034	<0.001	<0.001	-
Valine, leucine, and isoleucine degradation	9.62[b]	9.42[c]	9.87[a]	0.043	8.61[b]*	8.41[c]*	8.86[a]*	0.038	<0.001	<0.001	-
Carbohydrate Metabolism	11.2[b]	11.0[c]	11.4[a]	0.041	10.3[b]*	10.2[c]*	10.4[a]*	0.033	<0.001	<0.001	-
Amino sugar and nucleotide sugar metabolism	10.4[a]	10.1[b]	10.5[a]	0.040	9.42[a]*	9.16[b]*	9.52[a]*	0.032	<0.001	<0.001	-
Ascorbate and aldarate metabolism	9.64[b]	9.36[c]	9.87[a]	0.046	8.40[b]*	8.11[c]*	8.63[a]*	0.037	<0.001	<0.001	-
Butanoate metabolism	10.1[b]	9.87[c]	10.3[a]	0.040	9.03[b]*	8.82[c]*	9.25[a]*	0.033	<0.001	<0.001	-
C5-Branched dibasic acid metabolism	9.66[b]	9.44[c]	9.92[a]	0.041	8.73[b]*	8.51[c]*	8.99[a]*	0.035	<0.001	<0.001	-
Citrate cycle (TCA cycle)	9.99[a]	9.59[b]	10.2[a]	0.058	9.02[a]*	8.80[b]*	9.18[a]*	0.047	<0.001	<0.001	0.034
Fructose and mannose metabolism	10.2[a]	9.96[b]	10.4[a]	0.043	9.25[a]*	8.96[b]*	9.36[a]*	0.033	<0.001	<0.001	-
Galactose metabolism	10.0[a]	9.80[b]	10.1[a]	0.048	9.07[a]*	8.85[b]*	9.10[a]*	0.040	<0.001	<0.001	-
Glycolysis/Gluconeogenesis	10.3[a]	10.0[b]	10.4[a]	0.041	9.27[a]*	9.04[b]*	9.40[a]*	0.033	<0.001	<0.001	-
Glyoxylate and dicarboxylate metabolism	9.85[b]	9.58[c]	10.1[a]	0.041	8.98[b]*	8.71[c]*	9.24[a]*	0.034	<0.001	<0.001	-
Inositol phosphate metabolism	9.52[b]	9.06[c]	9.80[a]	0.059	8.27[a]*	8.08[b]*	8.43[a]*	0.047	<0.001	<0.001	0.006
Pentose and glucuronate interconversions	10.1[b]	9.82[c]	10.3[a]	0.043	8.97[b]*	8.73[c]*	9.22[a]*	0.037	<0.001	<0.001	-
Pentose phosphate pathway	10.1[b]	9.86[c]	10.2[a]	0.040	9.16[b]*	8.93[c]*	9.32[a]*	0.034	<0.001	<0.001	-
Propanoate metabolism	9.96[b]	9.78[c]	10.1[a]	0.046	8.91[b]*	8.73[c]*	9.03[a]*	0.046	<0.001	<0.001	-
Pyruvate metabolism	10.2[b]	9.99[c]	10.4[a]	0.040	9.23[b]*	9.01[c]*	9.37[a]*	0.035	<0.001	<0.001	-

Starch and sucrose metabolism	10.2[a]	9.94[b]	9.37[a]	0.041	9.23[a]*	8.89[b]*	9.37[a]*	0.035	<0.001	<0.001	-
Glycan Biosynthesis and Metabolism	10.6[b]	10.29[c]	10.8[a]	0.041	9.65[b]*	9.39[c]*	9.86[a]*	0.034	<0.001	<0.001	-
Glycosaminoglycan degradation	8.12	8.45	7.93	0.128	8.18[a]*	7.86[b]*	8.28[a]	0.067	0.878	0.479	<0.001
Glycosphingolipid biosynthesis - ganglio series	7.37	7.71	7.47	0.133	8.05[a]*	7.70[b]	8.15[a]*	0.066	0.573	<0.001	0.002
Glycosphingolipid biosynthesis - globo series	8.73[a]	8.55[b]	8.84[a]	0.054	8.30[a]*	8.12[b]*	8.41[a]*	0.053	<0.001	<0.001	-
Glycosyltransferases	9.89[b]	9.65[c]	10.1[a]	0.041	8.79[b]*	8.55[c]*	8.97[a]*	0.033	<0.001	<0.001	*
Lipopolysaccharide biosynthesis	9.71[a]	8.99[b]	10.0[a]	0.083	8.76[a]*	8.43[b]*	8.94[a]*	0.046	<0.001	<0.001	0.001
Lipopolysaccharide biosynthesis proteins	10.0[b]	9.46[c]	10.3[a]	0.058	8.90[a]*	8.59[b]*	9.09[a]*	0.055	<0.001	<0.001	0.005
N-Glycan biosynthesis	6.79	7.32	6.85	0.160	7.64[ab]*	7.46[b]	7.78[a]*	0.046	0.305	<0.001	0.005
Other glycan degradation	8.84	8.93	8.91	0.078	8.69[ab]*	8.46[b]*	8.78[a]*	0.074	0.156	<0.001	0.043
Peptidoglycan biosynthesis	10.1[a]	9.89[b]	10.2[a]	0.040	9.15[a]*	8.92[b]*	9.22[a]*	0.032	<0.001	<0.001	-
Various types of N-glycan biosynthesis	4.65	4.50	4.64	0.129	5.29*	5.14*	5.28*	0.106	0.668	<0.001	-
Metabolism of Other Amino Acids	10.8[b]	10.5[c]	11.0[a]	0.041	9.85[b]*	9.60[c]*	10.0[a]*	0.033	<0.001	<0.001	-
beta-Alanine metabolism	9.53[b]	9.04[c]	9.83[a]	0.059	8.53[a]*	8.28[b]*	8.69[a]*	0.054	<0.001	<0.001	0.010
Cyanoamino acid metabolism	9.64[b]	9.41[c]	9.80[a]	0.044	8.67[b]*	8.43[c]*	8.83[a]*	0.043	<0.001	<0.001	-
D-Alanine metabolism	9.39[a]	9.16[b]	9.49[a]	0.041	8.30[a]*	8.08[b]*	8.40[a]*	0.032	<0.001	<0.001	-
D-Arginine and D-ornithine metabolism	8.51[a]	7.94[b]	8.75[a]	0.066	7.02[ab]*	6.88[b]*	7.20[a]*	0.063	<0.001	<0.001	<0.001
D-Glutamine and D-glutamate metabolism	9.27[a]	9.04[b]	9.32[a]	0.040	8.40[a]*	8.17[b]*	8.45[a]*	0.033	<0.001	<0.001	-

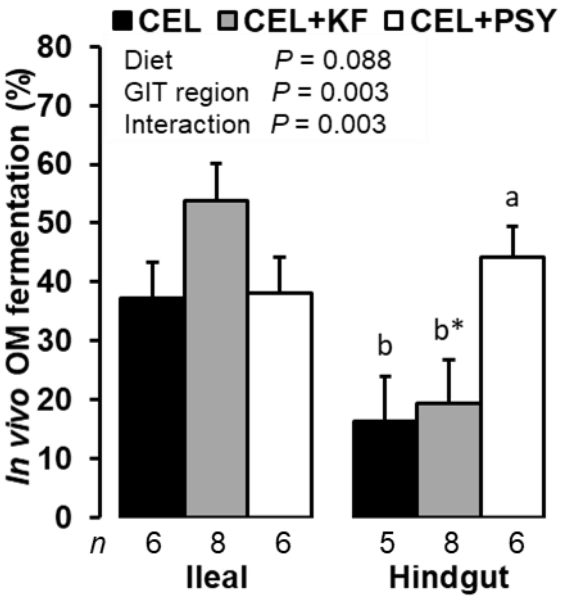
Glutathione metabolism	9.77[b]	9.52[c]	10.0[a]	0.043	8.60[b]*	8.34[c]*	8.83[a]*	0.036	<0.001	<0.001	-
Phosphonate and phosphinate metabolism	9.06[a]	8.85[b]	9.18[a]	0.048	7.99[a]*	7.78[b]*	8.11[a]*	0.047	<0.001	<0.001	-
Selenocompound metabolism	9.73[a]	9.49[b]	9.85[a]	0.040	8.81[a]*	8.57[b]*	8.93[a]*	0.032	<0.001	<0.001	-
Taurine and hypotaurine metabolism	9.17[a]	8.94[b]	9.28[a]	0.042	8.29[a]*	8.07[b]*	8.41[a]*	0.035	<0.001	<0.001	-
Digestive System											
Carbohydrate digestion and absorption	8.22	8.37	8.03	0.141	7.47[a]*	7.18[b]*	7.51[a]	0.066	0.745	<0.001	0.017
Protein digestion and absorption	6.49	6.88	6.68	0.149	7.66[a]*	7.33[b]	7.78[a]*	0.048	0.397	<0.001	0.005
Metabolism											
Amino acid metabolism	9.49[b]	9.26[c]	9.70[a]	0.041	8.59[b]*	8.36[c]*	8.81[a]*	0.034	<0.001	<0.001	-
Carbohydrate metabolism	9.23[a]	9.07[b]	9.37[a]	0.049	8.37[b]*	8.21[c]*	8.51[a]*	0.047	<0.001	<0.001	-
Glycan biosynthesis and metabolism	9.10[b]	8.40[c]	9.44[a]	0.064	7.94[a]*	7.66[b]*	8.11[a]*	0.061	<0.001	<0.001	<0.001

[1] Values are means with pooled SEM per GIT region. Data were $\log_{10}$ transformed to achieve homogenous variance before the two-way ANOVA test. The means within each GIT region with a different letter differ ($P \leq 0.05$), and the means with an asterisk for the faeces differ ($P \leq 0.05$) from their ileal digesta counterparts. CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PSY, psyllium.

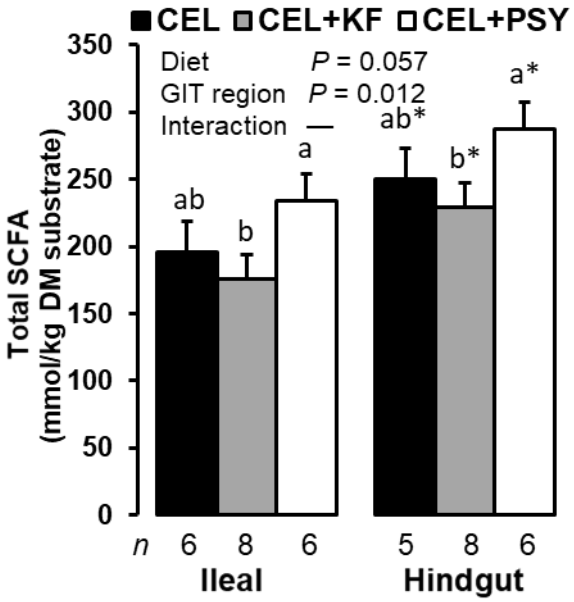
[2] When the interaction between the diet and GIT region was not significant ($P > 0.05$), this interaction was removed from the model.

[3] n indicates the number of replicates. The different numbers of replicates are due to insufficient digesta or faeces collected for analysis.

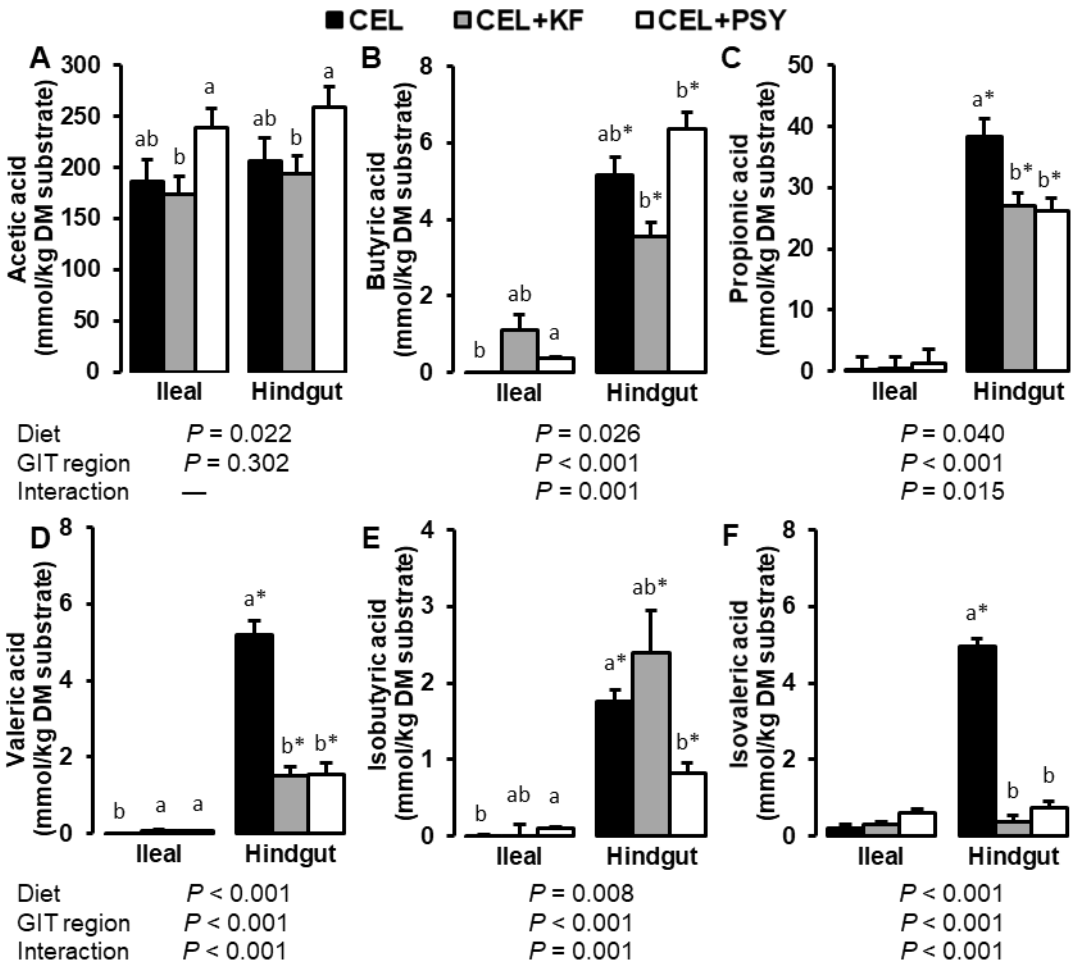
Supplementary figures



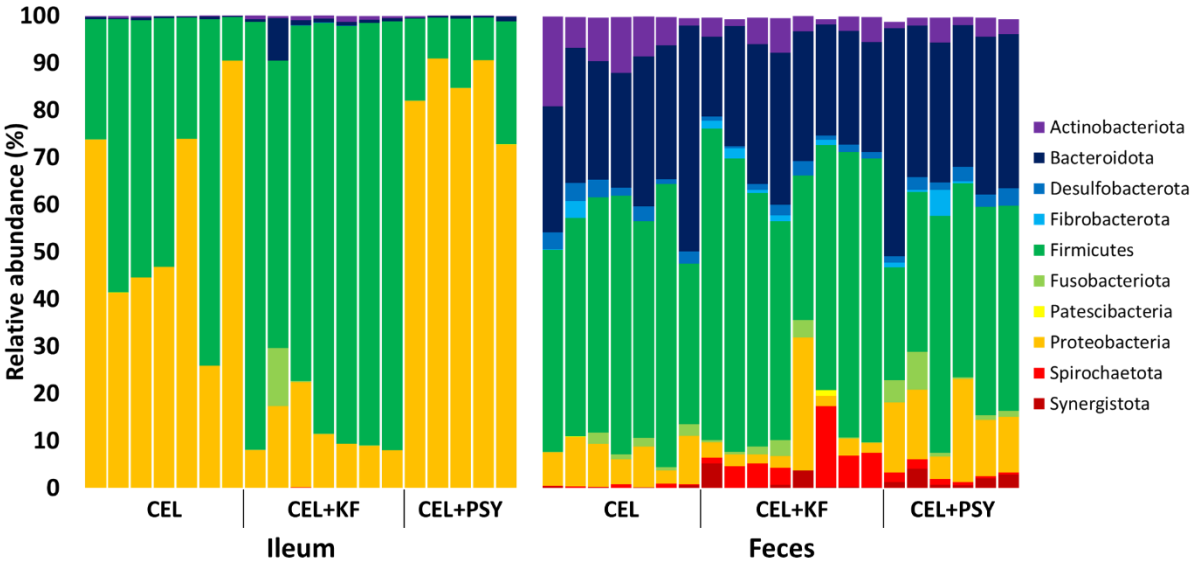
Supplemental Figure 6.1: Ileal and hindgut *in vivo* OM fermentation for pigs fed the CEL, KF, and PSY diets for 42 days. Values are mean ± SEM2, *n* indicates the number of replicates. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. Means within each GIT region with a different letter differ ($P \leq 0.05$). Means with an asterisk for the hindgut differ ($P \leq 0.05$) from their ileal counterparts. CEL, cellulose; GIT, gastrointestinal tract; KF, kiwifruit; OM, organic matter; PSY, psyllium.



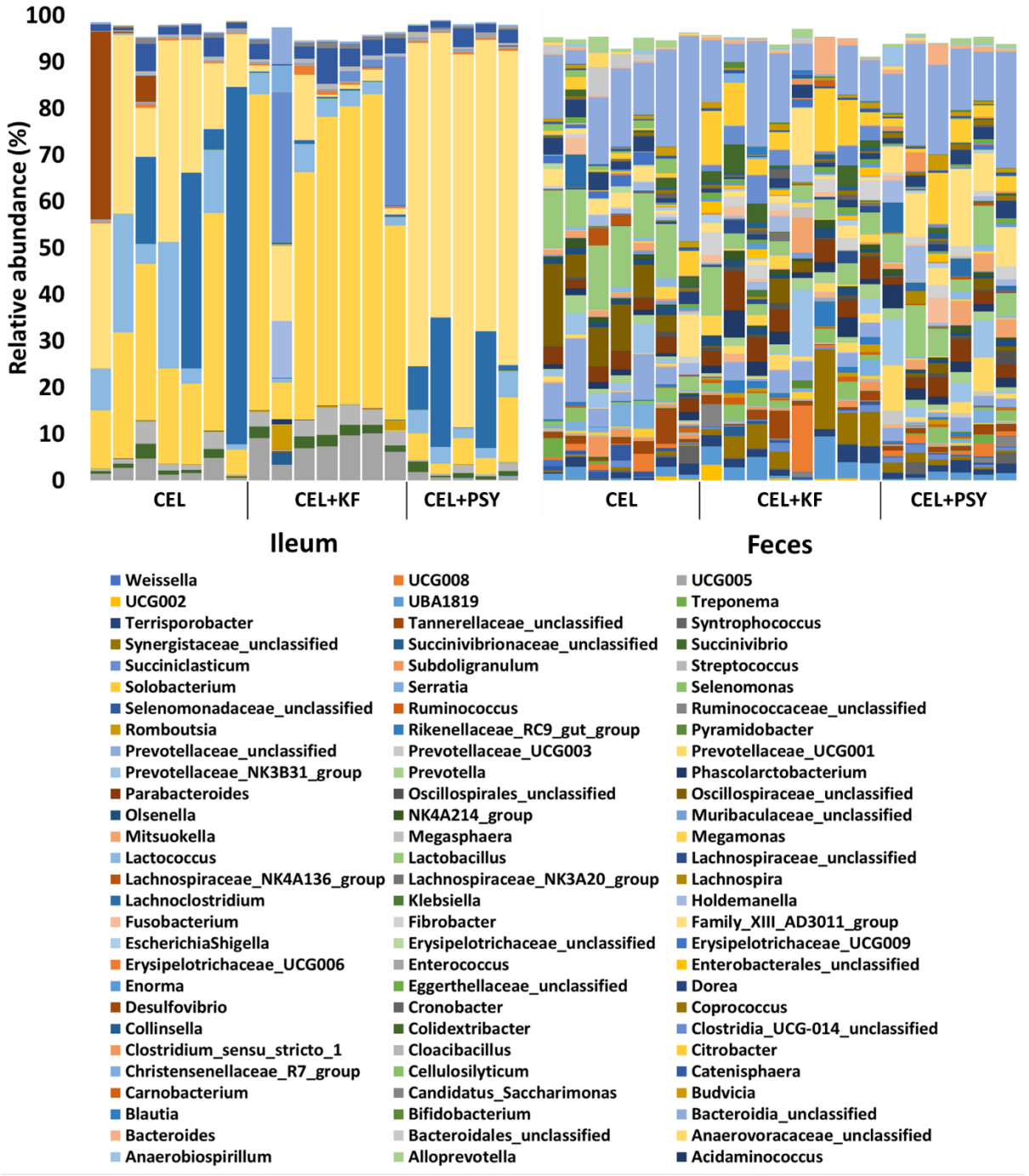
Supplemental Figure 6.2: Concentration of total SCFA after *in vitro* ileal and hindgut fermentation with ileal and caecal digesta, respectively, from pigs fed the CEL, KF, and PSY diets for 42 days. Values are mean ± SEM, *n* indicates the number of replicates. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. The interaction between diet and GIT region was not significant ($P > 0.05$) and therefore removed from the final model. Means within each GIT region with a different letter differ ($P \leq 0.05$). Means with an asterisk for the hindgut differ ($P \leq 0.05$) from their ileal counterparts. CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; PSY, psyllium; SCFA, short-chain fatty acids.



Supplemental Figure 6.3: Concentration of (A) acetic, (B) butyric, (C) propionic, (D) valeric, (E) iso-butyric, and (F) iso-valeric acids after *in vitro* ileal and hindgut fermentation with ileal and caecal digesta, respectively, from pigs fed the CEL, KF, and PSY diets for 42 days. Values are mean ± SEM, *n* indicates the number of replicates. A two-way ANOVA model was used to assess the effect of diet, GIT region, and the interaction between diet and GIT region. When the interaction between diet and GIT region was not significant ($P > 0.05$), it was removed from the final model. Means within each GIT region with a different letter differ ($P \leq 0.05$). Means with an asterisk for the hindgut differ ($P \leq 0.05$) from their ileal counterparts. When negative values were found (explained after removing SCFA found in the blanks), values are assumed to be zero (i.e., the SCFA was not produced). CEL, cellulose; DM, dry matter; GIT, gastrointestinal tract; KF, kiwifruit; PSY, psyllium; SCFA, short-chain fatty acids.



Supplemental Figure 6.4: Relative abundance of bacterial phyla in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for 42 days. Each column represents an individual replicate, *n* = 5-8 per diet. CEL, cellulose; KF, kiwifruit; PSY, psyllium.



Supplemental Figure 6.5: Relative abundance of bacterial taxa in ileal digesta and faeces of pigs fed the CEL, KF, and PSY diets for 42 days. Each column represents an individual replicate, *n* = 5-8 per diet. CEL, cellulose; KF, kiwifruit; PSY, psyllium.

Appendix 5

Chapter 7: Supplementary material

Supplemental methods

Ingredients and diet preparations

All test foods were food-grade and sourced commercially, except wheat bread. Pigeon peas, black beans, chickpeas, and sorghum were prepared at the Food Pilot Plant (Massey University) as described below, stored at -20°C, and defrosted before use. The non-protein-containing ingredients were mixed in the Feed Mill (Massey University) and stored at -20°C until use. The mix of non-protein-containing ingredients was specific to each diet (Supplementary Table 1). In addition, two indigestible markers, titanium dioxide (TiO_2; 4 g/kg DM) and celite (7.5 g/kg DM), were added to each diet, but they were not considered in this study. On days 6 and 7, the sorghum was prepared fresh before feeding. The wheat bran was prepared fresh for all meals.

Black Bean. Dried black beans were soaked in excess water (3:4 w/v ratio of dry beans to water) at room temperature for 18 h [384]. Black beans were drained. Table salt (720 mg per 100 g of soaked black beans) and water (1:1.15 w/v ratio of soaked black beans to water) was added before cooking in a commercial pressure retort (Mauri Engineering, Palmerston North, NZ) at 100 kPa and 121°C for 20 min.

Bread. The bread was baked at the Department of Food Science and Human Nutrition Pilot Processing Plant at the University of Illinois Urbana-Champaign, according to a standardised recipe. First, dry yeast and sucrose were combined with warm water. After it started foaming (i.e., yeast was active), salt, butter, flour, and the indigestible makers (titanium dioxide and celite) were added. Next, a commercial mixer (Hobart Legacy Mixer,

Troy, OH) was used to knead the bread dough. Then the dough was portioned into baking trays (23 x 13 x 6 cm) and allowed to rest for one hour. The bread was baked at 175°C for 35 minutes. The loaves of bread were then cooled to room temperature and frozen. Before feeding, the bread was thawed, sliced (13 mm slices), lightly toasted, and diced.

Chickpeas. Canned chickpeas (Sofia, Davis Food Ingredients) were drained and processed in a food processor for eight pulses over 15 seconds or until they reached a “chewed-like” texture.

Peanuts. Peanuts were sourced, de-shelled, and roasted. Roasted peanuts were coarsely ground.

Pigeon peas. Dried pigeon peas were prepared as described for the black beans, but with a cooking time of 10 min [384].

*Sorghum. S*orghum flour was prepared as a porridge with a final ratio of 1:4 flour to water (w/v). The sorghum flour was mixed with half the water before adding it to boiling water (i.e., the other half of the water). It simmered for 20 min while constantly stirring until it acquired a thick, porridge-like consistency [385].

Wheat bran. Wheat bran (Kellogg’s® All-Bran®) was mixed with water until the indigestible markers were homogeneously distributed.

Microbial analysis

The total number of bacteria (i.e., 16S rRNA gene copies) was determined using a quantitative PCR (qPCR). A qPCR instrument (LightCycler 480, Roche) was used to determine the total concentration of 16S rRNA gene copies in ileal digesta in duplicate [286]. SyBr Green detection chemistry (Roche) was used to amplify the DNA extracted for the ileal samples and the standard DNA (*Escherichia coli,* Nissle). The forward primer used was 5’-

TCCTACGGGAGGCAGCAGT, and the reverse primer was (5'-GGACTACCAGGGTATCTAATCCTGTT. Every qPCR run included an activation cycle (95°C, 5 min), 40 run cycles (i.e., denaturation (95°C, 30 s), annealing 60°C, 60 s) and extension (72°C, 60 s)) and one melting curve (60–95°C at 0·1°C/s). Data output was analysed using the LightCycler 480 Software (Version 1.5, Roche). The total number of 16S rRNA gene copies in the ileal digesta and the relative abundance of the taxa were used to determine the number of 16S rRNA gene copies per taxa with the assumption that each taxon has an equal number of 16s rRNA gene copies (calculation in Supplementary Methods).

Extracted DNA samples were sent to the Massey Genome Service (Massey University) for Illumina MiSeq sequencing to determine the taxonomic composition [286]. To amplify the V3-V4 hypervariable region of the 16S rRNA gene, the following primers were used: 16SF_V3 (5' - AATGATACGGCGACCACCGAGATCTACAC-index-TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). 16SF_V3 (5' - AATGATACGGCGACCACCGAGATCTACAC-index-TATGGTAATTGGCCTACGGGAGGCAGCAG) and 16SR_V4 (5' - CAAGCAGAAGACGGCATACGAGAT-index-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). The 96 libraries were prepared using the Illumina 16S V3-V4 rRNA library preparation method. The Massey Genome Service has dual index PCR primers which flank the V3-V4 hyper-variable region of 16S rRNA, which uses a Single Step PCR Library preparation method to prepare the libraries. The libraries were run on an Illumina MiSeq™ 2X 250 base PE, version 2 chemistry. In some samples,

the quality or concentration of the extracted DNA or both was too low to perform qPCR analysis and Illumina sequencing. Thus, the sample size per treatment differs.

The bioinformatics analysis was done using Mothur V1.44.2 (2, 3). Briefly, a total of 3,319,622 paired-end reads were detected. These reads were assembled and underwent quality control removing all reads with more than eight homopolymers and uncalled base. The average length of the sequences was 420 bp. Both the SLIVA database (version 138) (4) and the Greengenes database (version 13_8) (5) were used to align the sequences. After alignment, sequences were pre-clustered (4 bp) to remove noise and reduce the effect of sequencing errors. Chimeras were removed using VSEARCH (6), and all non-bacterial sequences were excluded. The remaining 2,640,909 reads were clustered into OTUs with a 97% cut off. A subsample of 47,642 reads per sample was used. The BIOM table generated after alignment with the SILVA database was used as input for Calypso (version 8.84) (7) to obtain the taxonomic composition, Shannon Diversity Index numbers and the principal coordinates analysis (PCoA) with the Bray-Curtis dissimilarities of the ileal microbiota. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (8) required the BIOM table generated after alignment with the Greengenes database to predict the metabolic activity of the ileal microbiota.

Calculations

The following equations were used to determine *in vitro* ileal OM fermentability [292]:

1) OM fermentability (%) = (OM $_{\text{before fermentation}}$ – [OM $_{\text{after fermentation}}$ – ((OM $_{\text{blank initial}}$ + OM $_{\text{blank final}}$)/2)]) / OM $_{\text{before fermentation}}$ x 100

where OM $_{\text{blank initial}}$ and OM $_{\text{blank final}}$ are the amounts of OM in the blanks before (initial) and after (final) *in vitro* fermentation, respectively. For each diet, the *in vitro* ileal fermentation had its own blanks.

The *in vitro* ileal production of organic acids was determined as described previously [292] using the following equations:

2) Organic acid production (mmol/kg substrate DM incubated) = (organic acid $_{\text{after fermentation}}$ (mmol) – [(organic acid $_{\text{blank initial}}$ + organic acid $_{\text{blank final}}$)/2]) / kg DM substrate

where organic acid $_{\text{blank initial}}$ and organic acid $_{\text{blank final}}$ are the organic acids (mmol) in the blanks before (initial) and after (final) *in vitro* fermentation, respectively. The *in vitro* ileal fermentation had its own blanks for each inoculum.

To determine the number of bacteria per taxa in ileal digesta, it was assumed that each taxon had an equal number of 16S rRNA gene copies. The number of bacteria per taxa in ileal digesta was calculated as follows [286]:

3) Number of bacteria per taxa$_{\text{ileal digesta}}$ (16S rRNA gene copy number/g wet digesta) = total number of bacteria$_{\text{ileal digesta}}$ (16S rRNA gene copy number/g wet digesta) x relative abundance $_{\text{taxa}}$ (%) / 100

Supplementary tables

Supplementary Table 7.1: The ingredient and determined nutrient composition of the diets

	Black bean	**Bread**	**Chickpea**	**Peanut**	**Pigeon pea**	**Sorghum**	**Wheat bran**
Ingredient, g/kg DM[1]							
Test food	448	946	522	357	488	942	803
Maize starch	314	-	244	405	274	-	-
Cellulose	30	-	30	30	30	-	71
Rapeseed oil	50	-	50	50	50	-	71
Sucrose	100	-	100	100	100	-	-
Vitamin/mineral mix[2]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Dicalcium phosphate	25	25	25	25	25	25	25
Calcium bicarbonate	3	3	3	3	3	3	3
Potassium bicarbonate	10	10	10	10	10	10	10
Sodium bicarbonate	3	3	3	3	3	3	3
Sodium chloride	4	-	-	4	4	4	1
Titanium dioxide	4	4	4	4	4	4	4
Celite	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Nutrient, g/kg DM[1]							
Ash	65.6	79.3	65.6	54.2	62.8	62.9	75.1
Crude protein	112	114	110	113	131	95	110
Total lipids	78.0	82.1	113	265	79.5	49.8	123
Starch	431	535	440	396	436	648	230
Total dietary fibre	176	57.2	129	57.0	144	72.6	295
- Insoluble fibre	146	46.8	121	53.4	140	67.7	278
- Soluble fibre	30	11.4	8.8	4.15	5.23	4.87	16.0
Insoluble:soluble fibre ratio	4.90	4.09	13.8	12.9	26.8	13.9	17.4
Gross energy MJ/kg	17.4	16.9	18.0	20.9	17.1	16.3	18.3

[1] DM, dry matter

[2] The vitamin and mineral premix supplied (per kg DM diet): Ca, 230 mg; Cu, 5.1 ppm; I, 0.6 ppm; Fe, 62 ppm; Mn, 30 ppm; Se, 0.15 ppm; Zn, 50 ppm; niacin, 22 mg; cobalamin, 0.02 µg; pantothenic acid, 12 mg; riboflavin, 3.3 mg; menadione, 0.7 mg; biotin, 0.2 mg; retinyl acetate, 1.7 µg; cholecalciferol, 0.03 µg; tocopheryl acetate, 32 µg; pyridoxine, 0.12 mg; folate, 0.79 mg; thiamine, 0.12 mg.

Supplementary Table 7.2: Overview of the ileal digesta samples collected during the experimental periods with the allocated diet for that pig[1]

Period	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7	Pig 8	Pig 9	Pig 10	Pig 11	Pig 12	Pig 13
1	-	-	PP	BB	WB	-	-	S	B	-	P	CP	-
2	-	PP	-	WB	-	-	S	B	P	-	CP	-	-
3	-	-	-	PP	BB	S	WB	-	-	-	B	P	CP
4	Protein-free diet												
5	Basal diet												
6	PP	BB	WB	-	S	B	P	CP	-	-	-	-	-
7	S	-	-	-	PP	BB	CP	WB	-	B	-	-	P
8	BB	WB	-	S	B	P	-	PP	-	CP	-	-	BB
9	-	S	-	-	-	PP	-	P	-	-	-	-	-

[1]A line represents no digesta sample collected from that pig during that period. B, bread; BB, black beans; CP, chickpeas; P, peanuts; PP, pigeon peas; S, sorghum; WB, wheat bran

Supplementary Table 7.3: Production of propionic and succinic acids during *in vitro* fermentation of dietary fibre substrates using pooled ileal inocula from growing pigs fed diets containing different test foods[1]

Organic acid	Inoculum	Substrate						Mean (range)
		AG	Cellulose	FOS	Inulin	Pectin	High-amylose starch	
		mmol/kg DM substrate						
Propionic acid	Black bean	0.376 ± 0.235[b]	ND	2.92 ± 0.263[a]	0.343 ± 0.304[b,§]	3.83 ± 0.304[a,†]	2.24 ± 0.263[a,‡]	1.64 (0.00 - 5.12)
	Bread	3.04 ± 0.946	1.24 ± 1.09[†‡]	3.34 ± 1.09	4.44 ± 0.846[‡]	ND	6.94 ± 1.34[†‡]	3.17 (0.00 - 6.96)
	Chickpea	ND	4.66 ± 0.581[†]	ND	ND	ND	ND	0.777 (0.00 - 6.20)
	Peanut	2.32 ± 0.465	1.68 ± 0.465[†‡]	2.25 ± 0.465	ND	3.40 ± 0.416[†]	1.91 ± 0.465[‡§]	1.93 (0.00 - 5.44)
	Pigeon pea	ND	1.46 ± 0.156[‡]	1.59 ± 0.180	ND	ND	0.855 ± 0.156[§]	0.737 (0.00 - 2.14)
	Sorghum	ND	ND	ND	ND	0.813 ± 0.134[‡]	ND	0.286 (0.00 - 1.65)
	Wheat bran	ND	ND	ND	8.49 ± 0.272[†]	ND	6.72 ± 0.352[†]	2.65 (0.00 - 9.87)
	Mean (range)	0.894 ± 0.169 (0.00 - 5.24)	1.36 ± 0.188 (0.00 - 6.20)	1.49 ± 0.187 (0.00 - 5.10)	1.95 ± 0.157 (0.00 - 9.87)	1.20 ± 0.188 (0.00 - 5.44)	2.69 ± 0.219 (0.00 - 7.60)	
Succinic acid	Black bean	ND	ND	ND	ND	ND	ND	
	Bread	5.67 ± 0.533[†]	ND	ND	ND	ND	ND	
	Chickpea	6.31 ± 0.477[†]	ND	ND	ND	ND	ND	
	Peanut	5.99 ± 0.477[†]	ND	ND	ND	ND	ND	
	Pigeon pea	1.50 ± 0.533[‡]	ND	ND	ND	ND	ND	
	Sorghum	0.23 ± 0.533[‡]	ND	ND	ND	ND	ND	

	Wheat bran	2.23 ± 0.447‡	ND	ND	ND	ND	ND
P value		Inoculum (I)	Substrate (S)	I x S			
	Propionic acid	<0.001	<0.001	<0.001			
	Succinic acid	<0.001	-	-			

[1] Values are means ± SEM, *n* = 5 fermentation bottles. A two-way ANOVA model was used to assess the effect of inoculum, substrate, and their interaction for propionic acid. A different repeated statement was required to have similar studentised residuals, described in the statistical analysis section. Means in a row (i.e., inoculum effect) with different letters differ ($P \leq 0.05$), and means in a column (i.e., substrate effect) with different symbols differ ($P \leq 0.05$). AG, arabinogalactan; DM, dry matter; FOS, fructooligosaccharides; ND, not detected.

Supplementary Table 7.4: Frequency of occurrence of taxa in ileal digesta from growing pigs fed diets for seven days containing different test foods[1]

Phylum	Genus	Diet							SEM	*P* value
		Black bean	Bread	Chickpea	Peanut	Pigeon pea	Sorghum	Wheat bran		
Sample size, *n*[2]		4	5	4	5	6	6	6		
Actinobacteria		1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Actinomyces	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Bifidobacterium	1.00[a]	0.500[b]	1.00[a]	0.600[ab]	0.857[ab]	0.500[b]	1.00[a]	0.024	0.050
	Collinsella	0.800[ab]	1.00[a]	0.600[ab]	1.00[a]	1.00[a]	0.500[b]	1.00[a]	0.017	0.050
Bacteroidetes		1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Alloprevotella	1.00	1.00	0.800	1.00	1.00	1.00	1.00	0.005	0.306
	Bacteroides	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Muribaculaceae_unclassified	1.00	0.750	0.600	0.800	0.857	1.00	0.833	0.024	0.118
	Parabacteroides	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Porphyromonas	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Prevotella	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Prevotellaceae_NK3B31_group	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Prevotellaceae_UCG003	1.00	1.00	0.800	1.00	1.00	1.00	1.00	0.005	0.306
	Prevotellaceae_unclassified	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Firmicutes		1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	Agathobacter	1.00	1.00	1.00	0.800	0.571	0.667	1.00	0.015	0.062
	Anaerovibrio	0.600[ab]	1.00[a]	1.00[a]	1.00[a]	0.857[ab]	0.500[b]	1.00[a]	0.015	0.050
	Blautia	1.00	1.00	0.800	1.00	0.857	1.00	1.00	0.007	0.153
	Cellulosilyticum	0.800	0.500	0.800	0.800	0.714	0.667	0.750	0.039	0.367

Clostridium_sensu_stricto_1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Enterococcus	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Faecalibacterium	0.600^{ab}	0.500^{b}	0.800^{ab}	0.600^{ab}	0.571^{ab}	0.500	1.00^{a}	0.038	0.050
Fusicatenibacter	0.600^{ab}	0.250^{bc}	0.200^{bc}	0.000^{c}	0.286^{bc}	0.167^{bc}	1.00^{a}	0.026	0.006
Lachnoanaerobaculum	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Lachnospiraceae_unclassified	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Lactobacillus	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Lactococcus	1.00	0.750	0.800	0.600	1.00	1.00	1.00	0.018	0.118
Leuconostoc	0.600^{ab}	0.250^{bc}	0.200^{bc}	0.200^{bc}	0.714^{ab}	0.833^{a}	0.000^{c}	0.030	0.036
Megamonas	1.00^{a}	0.750^{ab}	1.00^{a}	0.400^{b}	0.857^{ab}	0.333^{b}	1.00^{a}	0.021	0.013
Megasphaera	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	0.333^{b}	1.00^{a}	0.005	0.013
Mycoplasma	1.00	1.00	0.800	0.600	0.857	0.500	1.00	0.020	0.116
Parvimonas	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Phascolarctobacterium	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Romboutsia	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Sarcina	1.00	1.00	1.00	0.800	1.00	1.00	1.00	0.005	0.306
Selenomonadaceae_unclassified	1.00	1.00	1.00	1.00	1.00	0.833	1.00	0.003	0.315
Sharpea	0.200	0.000	0.200	0.200	0.429	0.000	0.00	0.019	0.062
Streptococcus	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Terrisporobacter	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Turicibacter	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Veillonella	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Weissella	0.800^{ab}	0.500^{bc}	0.400^{bc}	0.200^{c}	0.857^{ab}	1.00^{a}	1.00^{a}	0.027	0.004
Fusobacteria	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-

Fusobacterium	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Leptotrichia	1.00	1.00	0.800	1.00	1.00	0.667	0.75	0.017	0.134
Proteobacteria	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Acinetobacter	1.00^{a}	1.00^{a}	0.800^{ab}	0.600^{ab}	0.571^{b}	1.00^{a}	1.00^{a}	0.016	0.031
Actinobacillus	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Enterobacterales_unclassified	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Enterobacteriaceae_unclassified	1.00	1.00	0.800	1.00	1.00	1.00	1.00	0.005	0.306
Erwiniaceae_unclassified	0.200	0.000	0.400	0.000	0.143	0.833	0.00	0.017	0.002
Escherichia-Shigella	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Kosakonia	0.600^{ab}	0.500^{abc}	0.000^{c}	0.200^{bc}	0.714^{ab}	1.00^{a}	0.250^{bc}	0.031	0.013
Pantoea	0.600	0.250	0.600	0.400	0.714	0.833	0.50	0.044	0.070
Pasteurellaceae_unclassified	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Peptostreptococcaceae_unclassified	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Pseudomonas	0.200^{cd}	0.500^{abc}	0.000^{c}	0.400^{bc}	0.714^{ab}	1.00^{a}	0.250^{c}	0.031	0.034
Rahnella1	0.200^{b}	0.000^{b}	0.000^{b}	0.200^{b}	0.429^{ab}	0.833^{a}	0.000^{b}	0.017	0.002
Sutterella	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
Yersiniaceae_unclassified	0.200^{ab}	0.000^{b}	0.000^{b}	0.000^{b}	0.286^{ab}	0.667^{a}	0.000^{b}	0.014	0.013
Spirochaetes	0.800	0.750	0.800	0.600	0.571	0.667	1.00	0.033	0.062
Treponema	0.800	0.750	0.800	0.600	0.571	0.667	1.00	0.033	0.062
Synergistetes	1.00	1.00	1.00	1.00	1.00	1.00	0.750	0.007	0.292
Fretibacterium	1.00	1.00	1.00	1.00	1.00	1.00	0.750	0.007	0.292

[1] Values are frequencies with pooled SEM, *n* = 4-6 animals per diet. A value of 1 represents 100% frequency, and 0 represents 0% frequency. Only taxa with >1% relative abundance in at least one sample were considered. The frequency analysis was performed using a binary logistic regression with 0 when the taxon was absent and 1 when the taxon was present. No statistical analysis was performed if the frequency was 1 for all diets. Frequencies in a row with a different letter differ ($P \leq 0.05$).

[2] *n* indicates the number of replicates. The different number of replicates resulted from either removing one pig that displayed coprophagy or the extracted DNA having low quality or concentration for 16S rRNA gene sequencing.

Supplementary Table 7.5: Effect of diet on the number of bacteria in ileal digesta from growing pigs fed diets containing different test foods[1]

Phylum	Genus	Diet							SEM	*P* value
		Black bean	Bread	Chickpea	Peanut	Pigeon pea	Sorghum	Wheat bran		
Sample size, *n*[2]		4	5	4	5	6	6	6		
		Log_{10} 16S rRNA gene copies/g wet digesta								
Total bacteria		10.6^{ab}	11.1^{a}	10.8^{ab}	10.9^{ab}	11.1^{ab}	10.6^{b}	10.3^{b}	0.180	0.002
Actinobacteria		8.42	8.73	8.98	8.83	8.82	8.23	8.05	0.308	0.308
	Actinomyces	7.67^{ab}	8.32^{ab}	7.95^{ab}	7.82^{ab}	8.30^{a}	7.66^{ab}	7.43^{b}	0.192	0.036
	Bifidobacterium	7.73^{a}	6.77^{ab}	8.29^{ab}	7.34^{ab}	7.71^{ab}	5.98^{b}	6.87^{ab}	0.556	0.009
	Collinsella	6.99^{ab}	7.61^{a}	6.68^{ab}	7.74^{ab}	7.11^{ab}	6.04^{b}	7.47^{a}	0.357	0.010
Bacteroidetes		9.79^{b}	10.3^{a}	9.87^{ab}	10.3^{ab}	10.2^{ab}	9.59^{ab}	9.83^{ab}	0.177	0.021
	Alloprevotella	7.95	7.82	7.12	7.89	8.15	7.95	7.90	0.430	0.710
	Bacteroides	9.29^{ab}	9.79^{a}	9.33^{ab}	9.81^{ab}	9.67^{ab}	9.17^{ab}	8.64^{b}	0.229	0.038
	Muribaculaceae_unclassified	7.01	6.71	6.40	7.34	7.02	6.40	6.58	0.417	0.606
	Parabacteroides	7.47^{b}	8.23^{a}	7.50^{abc}	7.79^{abc}	7.92^{abc}	7.89^{ab}	6.73^{c}	0.252	0.001
	Porphyromonas	7.82	8.58	7.85	8.04	8.89	8.21	7.32	0.491	0.351
	Prevotella	9.29^{ab}	9.81^{a}	9.29^{ab}	9.45^{ab}	9.89^{ab}	8.93^{b}	9.71^{a}	0.205	0.009
	Prevotellaceae_NK3B31_group	7.61	8.22	7.81	7.97	7.89	7.33	7.90	0.490	0.908
	Prevotellaceae_UCG003	7.82	7.79	7.43	8.09	7.95	8.15	7.88	0.370	0.851
	Prevotellaceae_unclassified	8.85	9.30	8.72	9.17	9.16	8.52	8.52	0.241	0.139
Firmicutes		10.3^{ab}	10.9^{a}	10.6^{ab}	10.5^{ab}	10.8^{ab}	10.2^{b}	9.85^{b}	0.200	0.005
	Agathobacter	7.15^{ab}	7.52^{ab}	6.93^{ab}	7.28^{ab}	6.54^{ab}	6.14^{b}	7.86^{a}	0.352	0.027
	Anaerovibrio	6.66^{bc}	7.40^{b}	7.39^{ab}	8.09^{abc}	7.10^{abc}	5.74^{c}	8.82^{a}	0.398	<0.001

Blautia	7.25^{b}	7.44^{ab}	7.17^{ab}	7.81^{ab}	7.45^{ab}	7.18^{b}	8.47^{a}	0.329	0.008
Cellulosilyticum	7.79	7.51	6.48	8.34	7.08	7.06	6.27	0.484	0.068
Clostridium_sensu_stricto_1	8.54^{ab}	10.1^{a}	9.53^{ab}	9.62^{ab}	9.53^{ab}	8.98^{b}	8.13^{c}	0.309	0.035
Enterococcus	8.63^{a}	8.13^{a}	6.85^{b}	7.65^{ab}	8.47^{ab}	7.62^{a}	7.96^{ab}	0.379	0.007
Faecalibacterium	6.54^{bc}	6.47^{bc}	6.57^{bc}	6.57^{bc}	6.61^{b}	5.69^{c}	8.44^{a}	0.315	<0.001
Fusicatenibacter	6.08^{b}	6.14^{b}	5.84^{b}	ND	6.12^{b}	5.81^{b}	7.71^{a}	0.241	0.027
Lachnoanaerobaculum	7.66	8.19	7.69	7.57	8.05	7.60	7.78	0.256	0.523
Lachnospiraceae_unclassified	7.98^{b}	8.63^{a}	8.22^{ab}	8.71^{ab}	8.56^{ab}	8.30^{ab}	8.32^{ab}	0.192	0.019
Lactobacillus	7.59	8.31	8.86	7.66	8.37	8.64	7.93	0.618	0.706
Lactococcus	7.37^{ab}	6.66^{ab}	6.84^{ab}	6.19^{ab}	7.85^{a}	7.97^{a}	5.79^{d}	0.432	0.007
Leuconostoc	6.95	6.13	6.43	6.10	6.97	7.47	ND	0.453	0.093
Megamonas	7.62^{ab}	7.77^{abc}	7.95^{ab}	6.79^{bc}	7.63^{abc}	5.96^{c}	8.79^{a}	0.371	<0.001
Megasphaera	7.02^{ab}	7.14^{a}	8.19^{ab}	7.32^{ab}	7.67^{a}	5.67^{b}	6.82^{a}	0.486	0.001
Mycoplasma	7.60	8.25	6.88	7.11	7.59	6.53	7.10	0.505	0.315
Parvimonas	7.19	7.89	7.40	7.59	7.81	7.57	6.97	0.289	0.331
Phascolarctobacterium	8.29	9.11	8.62	9.14	8.86	8.25	8.74	0.223	0.063
Romboutsia	8.61^{abc}	9.81^{a}	9.26^{ab}	9.24^{ab}	9.41^{ab}	9.07^{b}	7.24^{c}	0.236	0.001
Sarcina	9.09^{ab}	10.3^{a}	8.37^{ab}	8.18^{ab}	9.91^{a}	8.30^{ab}	6.54^{b}	0.554	0.003
Selenomonadaceae_unclassified	7.33^{abc}	9.35^{abc}	9.66^{a}	7.37^{c}	9.43^{ab}	7.42^{abc}	7.70^{bc}	0.376	0.019
Sharpea	5.68	ND	6.58	6.06	6.42	ND	ND	0.415	0.132
Streptococcus	9.45^{ab}	9.80^{a}	9.31^{ab}	9.22^{ab}	9.49^{ab}	9.23^{ab}	8.75^{b}	0.290	0.039
Terrisporobacter	8.60^{ab}	9.62^{a}	9.26^{a}	8.91^{a}	8.63^{ab}	8.87^{a}	7.16^{b}	0.330	<0.001
Turicibacter	9.13^{ab}	9.52^{a}	9.54^{a}	9.67^{a}	9.45^{a}	8.68^{a}	7.58^{b}	0.324	<0.001
Veillonella	8.65^{ab}	9.19^{ab}	9.28^{a}	8.77^{ab}	9.36^{ab}	8.11^{b}	8.78^{ab}	0.297	0.030
Weissella	7.92	6.59	6.68	6.06	7.58	7.22	6.41	0.505	0.194

Fusobacteria	9.43^{b}	10.0^{a}	9.37^{b}	10.0^{ab}	9.79^{ab}	9.36^{b}	9.16^{b}	0.166	0.002
Fusobacterium	9.42^{b}	10.0^{a}	9.36^{b}	10.0^{ab}	9.76^{ab}	9.35^{b}	9.16^{b}	0.164	0.002
Leptotrichia	6.73	7.35	7.13	6.77	7.92	6.28	6.19	0.453	0.085
Proteobacteria	10.0^{ab}	10.1^{a}	9.59^{ab}	10.3^{ab}	10.2^{ab}	10.0^{a}	9.56^{b}	0.184	<0.001
Acinetobacter	7.28^{ab}	7.32^{ab}	6.24^{b}	6.77^{ab}	6.87^{ab}	8.31^{a}	6.73^{ab}	0.293	0.009
Actinobacillus	8.87	8.80	8.49	9.15	8.24	8.89	8.59	0.307	0.535
Enterobacterales_unclassified	7.58^{ab}	7.82^{a}	7.32^{ab}	7.80^{ab}	7.84^{ab}	7.91^{ab}	7.12^{b}	0.223	0.013
Enterobacteriaceae_unclassified	8.51	8.44	7.45	7.56	8.26	8.65	7.71	0.351	0.095
Erwiniaceae_unclassified	5.83	ND	5.90	ND	6.04	7.09	ND	0.367	0.372
Escherichia-Shigella	9.76	9.87	9.42	10.0	9.84	9.36	9.34	0.273	0.380
Kosakonia	6.99^{ab}	6.46^{ab}	ND	6.06^{ab}	7.36^{ab}	7.43^{a}	5.36^{b}	0.458	0.009
Pantoea	6.02	6.28	5.91	6.19	6.51	6.91	5.70	0.321	0.341
Pasteurellaceae_unclassified	8.08	8.44	8.03	8.14	8.04	7.82	8.17	0.321	0.917
Peptostreptococcaceae_unclassified	7.90	8.65	7.54	8.06	7.81	8.40	6.96	0.357	0.080
Pseudomonas	5.77^{bc}	6.26^{b}	ND	6.22^{bc}	6.49^{ab}	8.50^{a}	5.37^{c}	0.269	0.001
Rahnella1	6.04	ND	ND	6.10	6.28	7.47	ND	0.430	0.310
Sutterella	7.86^{ab}	8.43^{ab}	8.11^{ab}	8.67^{a}	8.31^{ab}	7.19^{b}	8.17^{ab}	0.326	0.035
Yersiniaceae_unclassified	5.84	ND	ND	ND	6.10	6.87	ND	0.038	0.296
Spirochaetes	7.04	6.98	6.51	7.08	6.84	6.90	6.65	0.519	0.986
Treponema	7.04	6.98	6.51	7.08	6.83	6.90	6.64	0.519	0.986
Synergistetes	7.16	7.98	7.41	7.58	7.86	7.47	6.80	0.397	0.476
Fretibacterium	7.11	7.89	7.39	7.29	7.85	7.43	6.80	0.386	0.467

[1] Values are means with pooled SEM, *n* = 4-6 animals per diet. Only taxa with >1% relative abundance in at least one sample were considered. The number of 16S rRNA gene copies per taxa was obtained by multiplying the total number of 16S rRNA gene copies with the relative abundance of the taxa with the assumption that each taxon has an equal number of 16S rRNA gene copies. Data were $\log_{10}$ transformed to achieve homogenous variance. A one-way ANOVA model was used to assess the effect of diet. Means in a row with a different letter differ ($P \leq 0.05$). ND, not detected.

[2] *n* indicates the number of replicates. The different number of replicates resulted from either removing one pig that displayed coprophagy or the extracted DNA having low quality or concentration for 16S rRNA gene sequencing.

Supplementary Table 7.6: Predicted metabolic activity (according to PICRUSt) related to fermentation (i.e., carbohydrate and protein metabolism) in ileal digesta from growing pigs fed diets for seven days containing different test foods[1]

KEGG reference pathway	Diet							SEM	*P* value
	Black bean	Bread	Chickpea	Peanut	Pigeon pea	Sorghum	Wheat bran		
Sample size, n[2]	4	5	4	5	6	6	6		
	log_{10} relative activity/g wet digesta								
Amino Acid Metabolism	6.96	6.95	6.96	6.94	6.86	7.03	6.96	0.049	0.260
Alanine, aspartate, and glutamate metabolism	5.96	5.97	6.97	6.97	5.88	6.02	6.00	0.044	0.314
Amino acid related enzymes	6.14	6.15	6.16	6.14	6.07	6.19	6.18	0.044	0.437
Arginine and proline metabolism	6.03	6.03	6.00	5.99	5.92	6.13	6.00	0.055	0.191
Cysteine and methionine metabolism	6.00	5.99	6.00	5.98	5.91	6.05	6.00	0.045	0.362
Glycine, serine, and threonine metabolism	5.92	5.92	5.91	5.89	5.82	6.00	5.93	0.051	0.251
Histidine metabolism	5.71	5.72	5.75	5.74	5.61	5.76	5.70	0.046	0.206
Lysine biosynthesis	5.83	5.84	5.86	5.82	5.75	5.88	5.86	0.046	0.377
Lysine degradation	5.27^{ab}	5.23^{ab}	5.23^{ab}	5.22^{ab}	5.12^{b}	5.44^{a}	5.16^{ab}	0.068	0.032
Phenylalanine metabolism	5.29	5.25	5.23	5.20	5.14	5.43	5.25	0.075	0.164
Phenylalanine, tyrosine, and tryptophan biosynthesis	5.84	5.85	5.86	5.84	5.75	5.89	5.90	0.051	0.383
Tryptophan metabolism	5.47^{ab}	5.43^{ab}	5.45^{ab}	5.40^{ab}	5.35^{b}	5.63^{a}	5.37^{ab}	0.069	0.007
Tyrosine metabolism	5.60	5.57	5.59	5.55	5.49	5.69	5.53	0.052	0.134
Valine, leucine, and isoleucine biosynthesis	5.80	5.80	5.79	5.78	5.71	5.87	5.79	0.045	0.243
Valine, leucine, and isoleucine degradation	5.50^{ab}	5.42^{ab}	5.43^{ab}	5.44^{ab}	5.31^{b}	5.62^{a}	5.35	0.070	0.031

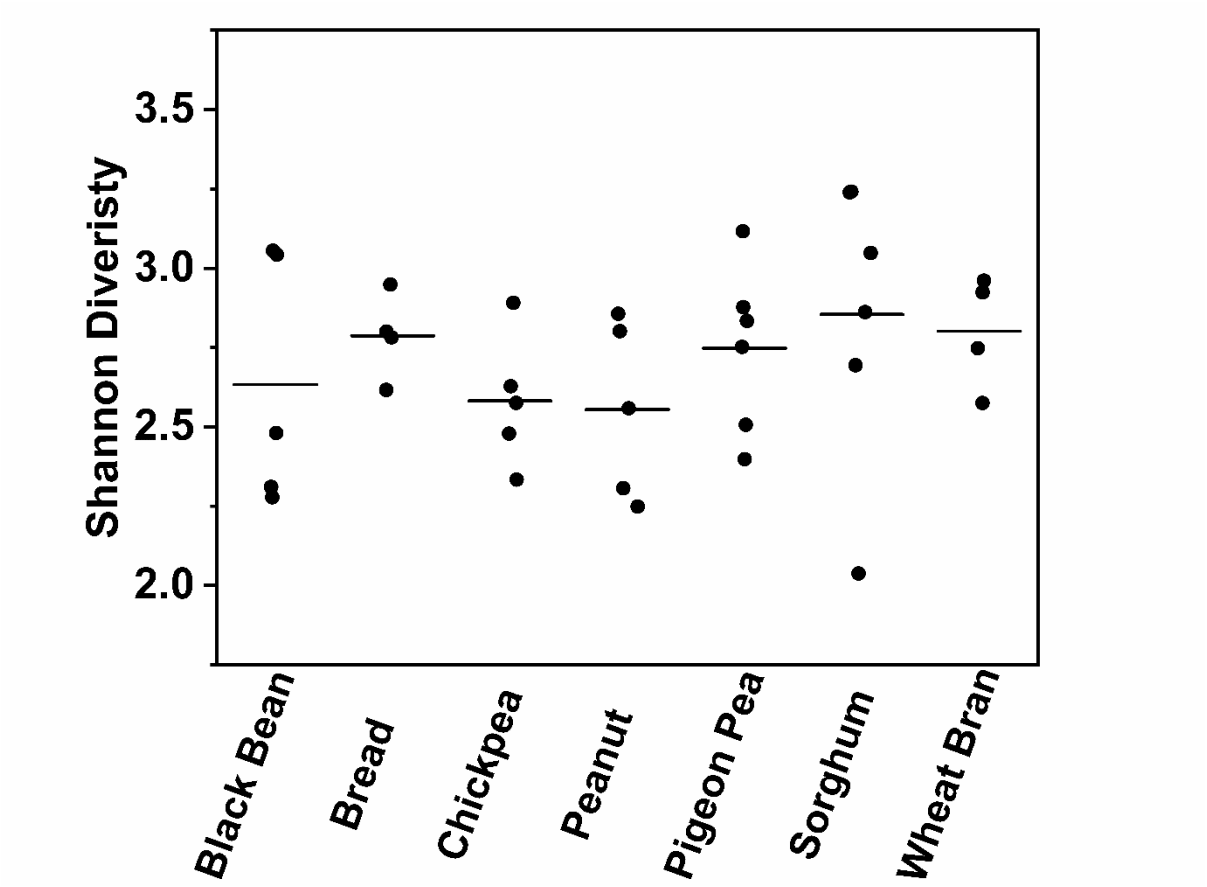
Carbohydrate Metabolism	7.03	7.01	7.00	6.98	6.93	7.10	7.00	0.047	0.192
Amino sugar and nucleotide sugar metabolism	6.17	6.16	6.14	6.13	6.09	6.21	6.17	0.041	0.470
Ascorbate and aldarate metabolism	5.33^{ab}	5.20^{ab}	5.08^{b}	5.20^{ab}	5.10^{b}	5.45^{a}	5.15^{ab}	0.093	0.040
Butanoate metabolism	5.88^{ab}	5.84^{ab}	5.84^{ab}	5.86^{ab}	5.72^{b}	5.96^{a}	5.78^{ab}	0.052	0.027
C5-Branched dibasic acid metabolism	5.44	5.45	5.45	5.43	5.35	5.53	5.48	0.050	0.211
Citrate cycle (TCA cycle)	5.80	5.77	5.77	5.77	5.71	5.86	5.84	0.056	0.479
Fructose and mannose metabolism	6.00	5.95	5.95	5.92	5.91	6.06	5.99	0.051	0.351
Galactose metabolism	5.84	5.84	5.78	5.77	5.75	5.88	5.80	0.038	0.186
Glycolysis/Gluconeogenesis	6.06	6.03	6.04	6.00	5.96	6.11	6.02	0.044	0.247
Glyoxylate and dicarboxylate metabolism	5.72^{ab}	5.67^{ab}	5.65^{ab}	5.69^{ab}	5.56^{b}	5.80^{a}	5.67^{ab}	0.057	0.050
Inositol phosphate metabolism	5.19^{ab}	5.04^{ab}	4.98^{ab}	5.10^{ab}	4.95^{b}	5.29^{a}	5.06^{ab}	0.088	0.047
Pentose and glucuronate interconversions	5.78^{ab}	5.71^{ab}	5.67^{ab}	5.72^{ab}	5.59^{b}	5.85^{a}	5.66^{ab}	0.052	0.009
Pentose phosphate pathway	5.91	5.89	5.89	5.86	5.81	5.98	5.88	0.047	0.211
Propanoate metabolism	5.76^{ab}	5.70^{ab}	5.71^{ab}	5.72^{ab}	5.60^{b}	5.84^{a}	5.67^{ab}	0.056	0.048
Pyruvate metabolism	6.03	6.00	5.99	5.97	5.92	6.10	5.96	0.049	0.177
Starch and sucrose metabolism	5.97	5.95	5.96	5.92	5.87	6.03	5.96	0.042	0.175
Metabolism of Other Amino Acids	6.21	6.19	6.20	6.17	6.10	6.29	6.19	0.051	0.198
beta-Alanine metabolism	5.30^{ab}	5.28^{ab}	5.26^{ab}	5.27^{ab}	5.19^{b}	5.47^{a}	5.36^{ab}	0.067	0.049
Cyanoamino acid metabolism	5.44	5.40	5.40	5.39	5.30	5.50	5.39	0.053	0.170
D-Alanine metabolism	5.09	5.08	5.09	5.06	5.00	5.12	5.07	0.039	0.416
D-Arginine and D-ornithine metabolism	3.72	3.80	3.91	3.70	3.71	3.83	3.93	0.092	0.403
D-Glutamine and D-glutamate metabolism	5.16	5.18	5.16	5.16	5.08	5.20	5.17	0.047	0.565
Glutathione metabolism	5.53^{ab}	5.43^{ab}	5.46^{ab}	5.49^{ab}	5.33^{b}	5.60^{a}	5.41^{ab}	0.056	0.015

Phosphonate and phosphinate metabolism	4.82	4.79	4.73	4.77	4.66	4.92	4.65	0.016	0.058
Selenocompound metabolism	5.57	5.56	5.57	5.53	5.48	5.62	5.57	0.049	0.454
Taurine and hypotaurine metabolism	5.09	5.07	5.11	5.06	4.98	5.13	5.02	0.051	0.271
Digestive System	4.59^{ab}	4.68^{ab}	4.65^{ab}	4.60^{ab}	4.62^{b}	4.63^{ab}	4.86^{a}	0.075	0.008
Carbohydrate digestion and absorption	4.28	4.31	4.35	4.17	4.27	4.35	4.40	0.080	0.571
Protein digestion and absorption	4.01	4.24	4.19	4.25	4.14	4.00	4.49	0.107	0.100
Metabolism	6.46^{ab}	6.40^{ab}	6.36^{ab}	6.42^{ab}	6.28^{b}	6.51^{a}	6.37^{ab}	0.053	0.044
Amino acid metabolism	5.45	5.41	5.36	5.39	5.32	5.50	5.36	0.056	0.213
Carbohydrate metabolism	5.14	5.12	5.09	5.04	5.01	5.24	5.08	0.060	0.115

[1] Values are means with pooled SEM, n = 4-6 per diet. Data were $\log_{10}$ transformed to achieve homogenous variance. A one-way ANOVA model was used to assess the diet effect. Means in a row with a different letter differ ($P \leq 0.05$). PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

[2] n indicates the number of replicates. The different number of replicates resulted from either removing one pig that displayed coprophagy or the extracted DNA having low quality or concentration for 16S rRNA gene sequencing.

Supplementary figure



Supplementary Figure 7.1: Shannon diversity of the microbial community in ileal digesta from growing pigs fed diets for seven days containing different test foods. Data points represent individual samples. The line represents the mean, *n* = 4-6 per diet. The effect of the diet was assessed using a one-way ANOVA test (*P* = 0.665).

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Hoogeveen AME, Moughan PJ, de Haas ES, Blatchford P, McNabb WC, Montoya CA. Ileal and hindgut fermentation in the growing pig fed a human-type diet. Brit J Nutr. 2020:1-27.

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Anna Maria Elisabeth Hoogeveen was responsible for the in vivo study, designed and conducted the the in vitro study, analyzed the samples, performed bioinformatics, subjected data to statistical analysis and wrote the draft manuscript

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- The percentage of the manuscript/published work that was contributed by the candidate:

- Describe the contribution that the candidate has made to the manuscript/published work:

Anna Maria Elisabeth Hoogeveen was responsible for the in vivo study, designed and conducted the the in vitro study, analyzed the samples, performed bioinformatics, subjected data to statistical analysis and wrote the draft manuscript

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

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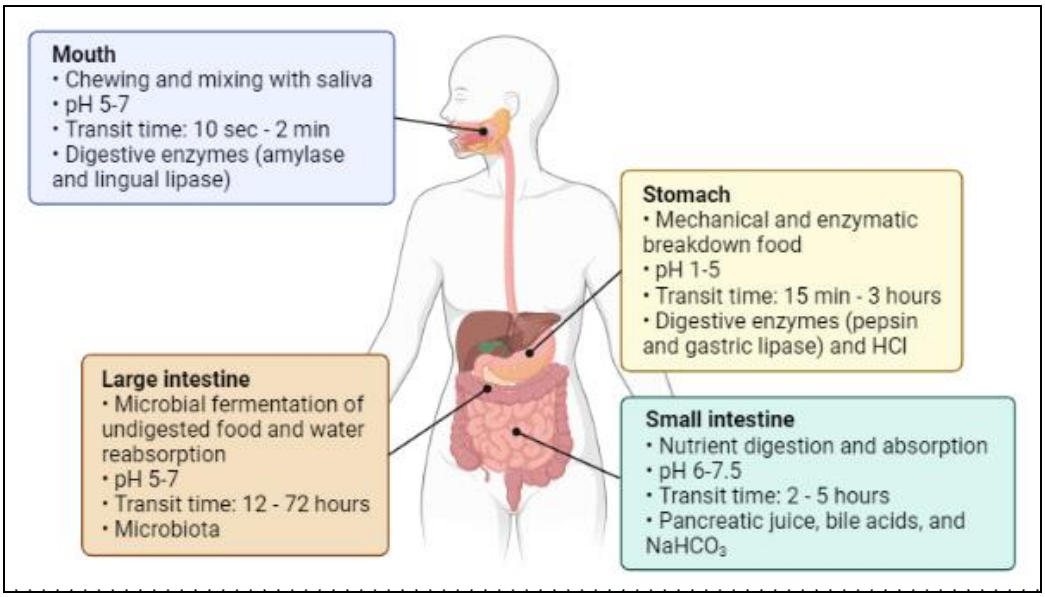

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