The effect of weaning age on gastrointestinal epithelial development of calves in New Zealand, a histomorphological analysis

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Abstract:

Dairy-beef calves born on New Zealand dairy farms are artificially reared and often weaned earlier than would occur under cow-calf beef production systems. Most weaning studies have focused on calves reared on concentrated diets for indoor systems and early (6-12 weeks of calf age) weaning systems. Weaning studies on artificially reared calves destined for grazing are limited and the effects of weaning age on gastrointestinal tract (GIT) development of dairy-beef calves reared on pasture are not known. This study evaluated the effects of milk-weaning at 10 (early) vs 20 (late) weeks of age on the histomorphology of the GIT of dairy-beef calves until 30 weeks of their age. The objectives of this study were to (1) evaluate differences in GIT epithelial development of early and late weaned calves and (2) evaluate whether differences (if any) persisted post-weaning. Male Hereford-Friesian calves (n=72) were sourced from two commercial dairy farms at three weeks of age and allocated to two treatments: early and late weaned. From the beginning of the study, all calves were kept outside (six calves per paddock; six paddocks per treatment) with free access to pasture (ryegrass clover mixed). A commercially purchased milk replacer (1 kg/day/calf; diluted in water to 7 L) was divided into two equal portions and fed twice daily using group feeders. Calves were weaned with a gradual reduction in milk replacer allowance beginning three weeks before designated weaning age. All calves were kept in their respective paddocks until humanely killed. Calves from each treatment were slaughtered across three different time points: 10 weeks, 20 weeks, and 30 weeks of age. At each time point 12 calves from each treatment (n=24) were slaughtered. Tissue samples from the rumen, duodenum, jejunum, and caecum were collected, processed to make histological slides, and analysed for histomorphological differences. The effect of slaughter age was significant for rumen ventral sac papillae length, duodenum villus width, jejunum crypt depth, and jejunum layer depth (essentially a combination measurement of villi length and crypt depth). The effect of weaning age was significant for caecum crypt depth, with early weaned calves having increased crypt depth across slaughter age. The interaction of weaning age and slaughter age was significant for rumen ventral sac papillae length, with early weaned calves having significantly greater papillae lengths ($P<0.05$) at slaughter week 20, but this difference did not persist post weaning (30 weeks). This study successfully reared and weaned calves at 10 and 20 weeks of age onto a pasture only diet without any major influence on histomorphology of their GIT observed.

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Chapter 1: General Introduction

The New Zealand dairy industry produced approximately 4.5 million calves in 2021 (Figure NZ 2022). It is estimated that approximately 28% are reared as replacements, another 12% are reared for dairy beef, and a further 15% are sold for dairy-beef rearing (Edwards *et al*. 2021). The remaining calves are bobby calves. These are excess calves, above requirements of the dairy and beef industry (Rodriguez Ferrere 2018) and are humanely slaughtered between 4-14 days old (MPI 2017). The dairy heifer calves and dairy beef calves are artificially reared in New Zealand and these artificial rearing conditions are very different to natural, dam-calf, conditions.

In New Zealand and elsewhere, beef (cow-calf) systems follow a rearing method that is almost analogous to that seen in natural conditions. These calves, have prolonged contact with, and suckling from the dam, typically until the calves reach their natural weaning age (Weary *et al*. 2008) of 6-9 months (Mikuš *et al*. 2020). Weaning under these conditions is usually a result of the "parent-offspring conflict", as the calf ages high maternal investment by the dam becomes less beneficial until suckling ceases (Weary *et al*. 2008). Typically, the dam will benefit more by offering maternal investment to new, younger, offspring (Weary *et al*. 2008). However, in dairy systems, calves are separated from the dam within 6-24 hours post birth (Verdon 2021) and fed colostrum. Calves are then fed milk or milk replacer (MR) via artificial rearing systems until weaning, usually 7-8 weeks of age (Schwarzkopf *et al*. 2019). Weaning under these conditions is a result of the coordinated removal of milk, involuntary to the calf.

There are many reasons for the earlier weaning age of artificially reared calves. Feeding milk or MR is often associated with high costs and early transition to solid feeds helps to reduce these costs (Khan *et al*. 2016). Calves, reared under natural/beef systems have many small feeds throughout the day (de Passillé *et al*. 2011). However, under artificial rearing conditions it is not economically viable to feed calves many times throughout the day, unless an automatic milk feeding system is being used (Muir 2022). Therefore, calves are usually offered milk once- or twice- a day (Moran 2012) and the milk-feeding period is minimised to reduce feed and labour costs associated with milk feeding. Furthermore, weaning age is a manipulation of artificial rearing conditions, rather than a natural process, and majority of the research is focused on early weaning system (4-12 weeks of calf age). Scientific literature on how weaning age may affect the development of the calf, particularly development of the gastrointestinal tract (GIT) is scarce. Furthermore, previous weaning studies have found contradictory evidence of the effect of weaning age and weaning method on GIT development.

In some studies, the effect of weaning age on rumen development has been found to have little-to-no effect on the rumen development of calves (Kehoe *et al*. 2007; Schwarzkopf *et al*. 2022) and lambs (Carballo *et al*. 2019a). However, other literature has found evidence of improved rumen development for early weaned

calves (Zitnan *et al*. 1999; Stobo *et al*. 1966; McCoard *et al*. 2019; Dong *et al*. 2019) and even, negative impacts of early weaning on rumen development were reported by Meale *et al*. (2017b). It is also reported that calves reach at 'mature' rumen state at around 11 weeks of age (Schwarzkopf *et al*. 2022; Abdelsattar *et al*. 2022; Jiao *et al*. 2015) and it is difficult to permanently change the rumen after it reach to maturity (Arshard *et al*. 2021). Literature on the effect of weaning age on the development of the lower GIT, which includes the small intestine, caecum, and large intestine, is also conflicting. In lambs, it has been reported that there is no difference in the structural development of the lower GIT for different weaning ages (McCoard *et al*. 2020). However, it has been reported that the microbial population of the lower GIT differs because of weaning age (Schwarzkopf *et al*. 2022; Osuka *et al*. 2012).

Overall, it is difficult to compare the results from various studies because rearing conditions in current published studies differ, not only weaning age, but also other pre-weaning and weaning factors such as milk allowance, type of milk (MR of various kinds, pasteurised or unpasteurised milk), milk composition, physical structure (mash, pelleted and textured) and type (concentrate and/or pasture) of the solid feeds. Further, weaning methods implemented also differ in the published studies. It is important to reiterate that most of the published studies on weaning age were conducted with calves reared in indoor systems and calves were weaned between 4-12 weeks of age. Further, calves in these studies were reared on pelleted and textured concentrate feeds with or without forage. Few studies have compared the effects of delaying the weaning beyond three months of calf age. There is a clear gap in scientific information on the effects of weaning age on epithelial development of calf GIT destined for forage grazing (e.g., New Zealand heifer and dairy-beef calves).

The development of the GIT is important due to its effect on the overall lifetime performance of the calf, whether it is destined for the dairy or beef industry. A well-developed GIT allows for efficient digestion and absorption of nutrients and early life nutrition has a major effect on lifetime performance of the cow (Byrne *et al*. 2022; Heinrichs and Heinrichs 2011; Burggraaf *et al*. 2020; Diao *et al*. 2019). It has been suggested that improvement of early life nutrition improves the adaptive immune response of cows (Byrne *et al*. 2022) and improves first lactation milk production (Fernando 2011 Heinrichs and Heinrichs 2011). Therefore, understanding how manipulations of weaning age may affect the development of the GIT will generate new knowledge to make scientifically informed calf management practices to enhance lifelong welfare and performance of artificially reared calves.

The objectives of this thesis were to investigate the histomorphological differences in the GIT of early (10 weeks of age) and late (20 weeks of age) weaned calves reared on pasture, and to evaluate whether these differences (if any) persisted post-weaning at 30 weeks of calf age. In an attempt to minimise differences due

to variation in concentrate intake, concentrate feeds were not provided to the calf and calves were reared only on a milk replacer and pasture diet. This would also help create study conditions more like the New Zealand rearing system. I hypothesised that the greatest difference, between early and late weaned calves will be seen at slaughter week 20, due to the consumption of different diets at this time. Further, I hypothesised that differences will be sustained at 30 weeks, but the differences will not be as large.

Chapter 2: Literature Review

2.1 Anatomy and function of the gastrointestinal tract at birth

At birth, the GIT of ruminant animals, including calves, is remarkably different and underdeveloped compared to the GIT of mature ruminants. This includes differences in the anatomical and physical features (Diao *et al*. 2019; Meale *et al*. 2017a), anaerobic microbial populations (Jami *et al*. 2013) and metabolic functions (Baldwin and Connor 2017). At birth, the digestive tract of calves lacks microbial diversity, the rumen is rudimentary, and the intestines are immature. Detailed information on the features and functions of the calf digestive tract is summarised below.

2.1.1 Oesophageal groove

The oesophageal groove is a key anatomical feature of the pre-ruminant digestive tract. The groove forms as two pillars from the cardiac end of the oesophagus, over the dorsal wall of the reticulum (Comline and Titchen 1951; Martín-Alonso *et al*. 2019), combine. Milk ingestion causes stimulation of nerve receptors in the mouth (Larry 2002) and as a result muscles in the pillar contract, forming a tube-like structure which allows liquids to pass directly into the abomasum (Baldwin *et al*. 2004). The key function of the oesophageal groove is to prevent milk entering the rumen, which may cause ruminal acidosis (Gentile 2004) and fatal bloat (Kaba *et al*. 2018). Suckling helps maintain the oesophageal groove and this feature disappeared in non-suckling calves after they were milk weaned (Kaba *et al*. 2018).

2.1.2 Rumen

The forestomach (rumen, reticulum, and omasum) of the calf is physically and metabolically underdeveloped at birth (Baldwin and Connor 2017). Rumen and reticulum make up 30% of the total forestomach capacity in newborn calves (Meale *et al*. 2017a; Warner *et al*. 1956; PennState Extension 2022). The undeveloped rumen also lacks a diverse microbial population (Jami *et al*. 2013). There is little muscle development and therefore little ruminal contractions (Pal *et al*. 2019). Further, the underdeveloped rumen has no papillae (Heinrichs 2005), keratinisation (Gilliland *et al*. 1962) or pigmentation (Tamate *et al*. 1962) of the epithelium, compared to mature ruminants. The rumen undergoes tremendous morphological, physiological, and microbial changes before it is completely functional (Jami *et al*. 2013) and can efficiently ferment solid feeds (Meale *et al*. 2017a). The rumen has no real functional significance in calves, until consumption of solid feed begins. Digestion continues in the abomasum and lower GIT, like that of a monogastric animal, explained below.

2.1.3 Abomasum

The abomasum makes up around 60% of the forestomach capacity in young ruminant animals (PennState Extension 2002). There are four layers of the epithelium; tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa (Aage *et al*. 2007; Masot *et al*. 2007). However, there is conflicting evidence as to whether the epithelium is stratified squamous (Aage *et al*. 2007) or pseudostratified (Masot *et al*. 2007). The main function of the abomasum is acidic digestion (Baldwin *et al*. 2004). The mucosal surface of the abomasum

has folds which increase surface area to aid digestion (Masot *et al*. 2007), cell type changes at the tip of the mucosal fold to tall columnar cells (Aage *et al*. 2007; Masot *et al*. 2007). The abomasum has gastric glands containing chief cells and parietal glands (Kitamura *et al*. 2001). The chief cells secrete pepsin (Wu 2017; Fish and Burns 2022) and rennin (in the form of pepsinogen and pro rennin, respectively) (Wu 2017), which causes milk to clot (Saha and Pathak 2021; Porter 1969). Pepsin also breaks down proteins (Parish *et al*. 2017). Pepsin and rennin require an acidic environment to function, this is provided by the secretion of hydrochloric acid (Hertzberg *et al*. 2000) from parietal cells in the gastric glands (Wu 2017). Lipase is also secreted to the abomasum from the pancreas (Parish *et al*. 2017; Wu 2017) and breaks down fats (Parish *et al*. 2017; Wu 2017). The abomasum, alongside the small intestine, are important for pre-ruminant animals as these are the key areas for digestion (Baldwin *et al*. 2004; Guilloteau *et al*. 2009a).

2.1.4 Lower gastrointestinal tract

The small intestinal wall has simple columnar epithelial tissue (Jennings and Premanandan 2017; Walton *et al*. 2018) comprised of four layers; the tunica mucosa, tunica submucosa, tunica muscularis propria, and tunica serosa (Jennings and Premanandan 2017). A distinguishing feature of the small intestine is the presence of many finger-like projections called villi (Walton *et al*. 2018; Streckfus 2022). Villi increase the surface area of the small intestine, thus functionally increasing the absorption of nutrients (Yang and Nam 2022; Streckfus 2022). The absorption of nutrients is crucial in terms of immunity. Immediately after birth the calf intestines can be considered “open” allowing for the ability to absorb large molecules, such as immunoglobulins from colostrum (Smeaton and Simpson- Morgan 1985; Louis and Lin 2009). These provide passive immunity to the newborn calf for up to 2-3 weeks (Fishcer *et al*. 2019; Heinrichs and Elizondo-Salazar 2009), before the active immune system takes over.

Enterocytes and goblet cells are present in the villi, these are responsible for the absorption of water/electrolytes and the secretion of mucus, respectively (Streckfus 2022). Villi run into ‘crypts of Lieberkühn’ (Yang and Nam 2022) which travel deep into the submucosa. These crypts contain many cells such as enterocytes, goblet cells, enteroendocrine cells, cup cells, tuft cells and Paneth cells, all with varying functions (Streckfus 2022). It appears that mammalian species are not born with the presence of crypts but develop them within the first week of life (Barker *et al*. 2008). Therefore, the function of the small intestine may be limited as cells present in the crypts will be absent. There also appears to be a negative relationship between crypt depth and villus height resulting in reduced villus height/crypt depth ratio (Steinhoff-Wagner *et al*. 2015).

The small intestine consists of three key areas each with varying functions (Lindholm-Perry *et al*. 2016; Yang and Nam 2022. The duodenum receives chyme and pancreatic secretions that continue chemical digestion

(Beaumont *et al*. 2021; Fish and Burns 2022). The following area is the jejunum, primarily responsible for nutrient absorption (Beaumont *et al*. 2021; Tappenden 2014) and finally the ileum which absorbs remaining nutrients (Beaumont *et al*. 2021; Gaowa *et al*. 2021) and vitamins (Beaumont *et al*. 2021; Park 2022).

The duodenum receives pancreatic proteases (such as trypsin, chymotrypsin, elastase, and pepsin) which help digest protein (Wu 2017; Harmon and Swanson 2020; Fish and Burns 2022), with help from the action of mucosal maltase (Harmon and Swanson 2020). The pancreas also secretes lipase (Wu 2017; Fish and Burns 2022), colipase (Wu 2017) and bile salts (Wu 2017; Perez-Barberia 2020) into the small intestine for fat digestion (Wu 2017; Perez-Barberia 2020). The main carbohydrate in the pre-ruminant diet is lactose from milk (Klinger *et al*. 2013), this has a digestibility of around 99% in the small intestine (Wu 2017; Gilbert 2015) due to the action of lactase (Kaushal *et al*. 2021) secreted from the microvilli of the small intestine (Ren *et al*. 2019). The Brunner cells present in the duodenum secrete mucin glycoproteins and small amounts of bicarbonate which help to neutralise the digesta that has just entered from the abomasum (Krasue 2000; Jennings and Premanandan 2017; Park 2022). These secretions are incorporated into the mucus layer, therefore, also having a protective function too (Krause 2000; Park 2022). Any digesta that is not absorbed in the small intestine passes into the large intestine.

The large intestine is comprised of the caecum, colon, rectum, and anus (Nigam *et al*. 2019; Kahai *et al*. 2023), its main function is absorption (Barker *et al*. 2008). It absorbs minerals (Barker *et al*. 2008; Nigam *et al*. 2019; Kahai *et al*. 2023; Perez-Barberia 2020) and vitamins (Kahai *et al*. 2023; Perez-Barberia 2020). It also functions to form faeces from remaining indigestible food materials (Barker *et al*. 2008; Nigam *et al*. 2019; Kahai *et al*. 2023). Like the small intestine, the large intestine has a simple columnar epithelium (Vaňhara 2020), with four layers; tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (Jennings and Premanandan 2017). However, while the large intestine has crypts of Lieberkühn, they lack the presence of villi (Barker *et al*. 2008; Nigam *et al*. 2009). The crypts of Lieberkühn contain goblet cells and enterocyte cells (Streckfus 2022). The depth of these crypts increases rapidly during the first week of life (Barker *et al*. 2009). The large intestine has a much higher density of goblet cells compared to the small intestine (Streckfus, 2022; Barker *et al*., 2008), especially dense in the colon (Kim and Ho, 2010; Bass and Wershill, 2020). The rectum also has transverse rectal folds (Streckfus 2022; Lee and Mezoff 2021). The anal canal changes in epithelium type from columnar to stratified cuboidal (Streckfus 2022).

At birth, the lower GIT has the presence of villi in the small intestine and crypts in the large intestine. In the first few weeks of life, the small intestine rapidly develops crypts, decreasing the villus height/crypt depth ratio. In the large intestines, crypts are present at birth and quickly increase in depth. The small intestine is

the key area for digestion in the calf, with many secretions to aid digestion. The large intestine functions to absorb.

In summary, during the first few months of life, various sections of the ruminant GIT undergo tremendous changes including the establishment of a stable anaerobic microbial population, the relative size of the upper and lower digestive tract, maturation of epithelium and absorption and secretory traits (Khan *et al*., 2016). These changes are influenced by the early-life management of young ruminants including nutrition, housing, and weaning.

2.2 Anatomy and function of the gastrointestinal tract of the mature ruminant

Ruminants have a multiple-stomach system comprised of the rumen, reticulum, and omasum (together representing the upper GIT), that allows them to efficiently ferment and use the organic plant matter they selectively consume (Choudhury *et al*. 2015; Giger-Reverdin *et al*. 2020) before peptic (abomasum, true stomach) and intestinal digestion. Features and functions (enzymatic secretion, nutrient absorption, protective barrier) of the lower GIT in ruminants are almost like monogastric animals (Gäbel *et al*. 2002; Steele *et al*. 2016) and change little in regard to the pre-ruminant digestive tract. Anatomical features (Figure 2.1) and function of the adult ruminant digestive tract are discussed below.

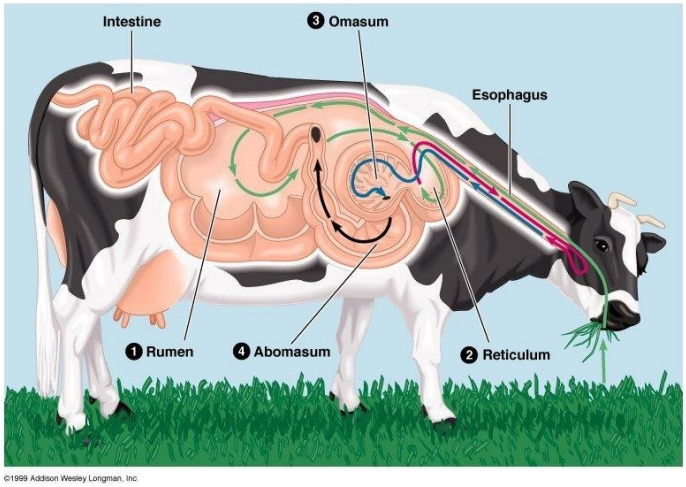

Figure 2.1 Adult ruminant digestive system (Campbell *et al*. 1999)

The oesophageal groove no longer functions in mature ruminants. After a few weeks of weaning, the response to stimulus reduces until the groove is no longer functional (Kaba *et al*. 2018). This allows feeds to enter the rumen and undergo ruminal fermentation.

With the initiation of solid feed, the rumen undergoes many morphological and physiological changes to reach a 'mature' rumen (Meale *et al*. 2017b). The capacity of the rumen is 70% (Meale *et al*. 2017b; Warner *et al*. 1956) to 80% (PennState Extension 2022) of the entire GIT capacity. The increase in size of the rumen allows it to be the key site of digestion compared to the abomasum (and small intestine) in pre-ruminants.

The rumen epithelium is composed of stratified squamous cells (Baldwin and Connor 2017; Steele *et al*. 2016) that have a protective function, as well as absorption, transport, and VFA metabolism functions (Baldwin and Connor 2017). There are four layers of the stratified squamous epithelium; the stratum basale is the first layer, laying adjacent to the basal lamina and stratum spinosum (Steele *et al*. 2016). The stratum basale and stratum spinosum layers both contain mitochondria which assist the ruminal epithelia's metabolic properties (Baldwin *et al*. 2004). Adjacent to the stratum spinosum is the stratum granulosum, distinguishable from other cellular layers by the presence of tight junctions, adhering junctions, and desmosomes (Steele *et al*. 2016). Collectively, these junctions increase the mechanical strength of the epithelium (Graham and Simmons 2005). Adjacent to the stratum granulosum is the stratum corneum which is in direct contact with the ruminal contents (Steele *et al*. 2016). The stratum corneum provides an important protective function by preventing microbes and toxic compounds perforating the epithelium (Baldwin and Connor 2017; Steele *et al*. 2016; Na and Guna 2022). Essentially, the stratum corneum acts as a physical barrier, like the function of the mucus layer of the lower GIT (Baaske *et al*. 2020). Although some ruminal bacterial populations colonise the surface of the stratum corneum, they do not reach the stratum granulosum (Graham and Simmons 2005). Papillae are leaf-like structures that project from the surface of the rumen epithelium, typically 10-15 mm in length (Graham and Simmons 2005). They are a key ruminal adaptation that increase the surface area of the epithelium and allow for more efficient absorption of fermentation end products (Heinrichs 2005). There are distinctive ridges and hollows on papillae, and under magnification, highly keratinised squamous cells can be observed (Graham and Simmons 2005). Often there are a substantial number of cells detaching from these ridges, suggesting that the outer keratinised layer doesn't act as a protective barrier to epithelial permeability (Graham and Simmons 2005).

The mature rumen also relies on muscle tissues for contractions that allow the rumen to empty (Fioramonti and Bueno 1988) and regulate the regurgitation process (Khan *et al*. 2016). The mature rumen muscle tissue has an extensive intrinsic nervous system that allows for coordinated motility patterns/ contractions (Herdt 2020). Greater muscularisation and thickening of the rumen wall allows for increases in the physical capacity of the rumen (Pazoki *et al*. 2017), which is particularly important for animals raised under pastoral systems due to the high bulk of pasture.

Ruminants consume a diet that cannot be utilised by the animal itself, instead the rumen share a symbiotic relationship with ruminal microbiota (Choudhury *et al*. 2015). Microbes actively breakdown carbohydrates in plants to produce energy for themselves as well as the ruminant (Moran 2012; Choudhury *et al*. 2015). The fermentation of these carbohydrates produces volatile fatty acids (VFAs) such as acetate, propionate, and butyrate (Graham and Simon 2005). VFAs are selectively absorbed from the rumen and used to produce energy, with more than 75% of them being absorbed in the rumen and reticulum (Church 1969), only 10% of ruminally produced VFAs reach the small intestine (Harfoot 1978). The production of VFA's is also important to support further development of the rumen, particularly the rumen epithelium (Flatt *et al*. 1958; Sander *et al*. 1959; Baldwin *et al*. 2004) and papillae (Sander *et al*. 1959; Tamate *et al*. 1962) due to a likely increase in concentration of butyrate. Butyrate is considered the main VFA responsible for rumen development (Sander *et al*. 1959; McLeod and Baldwin 2000; Castells *et al*. 2012).

There is conflicting evidence as to when microbial colonisation begins. Meale *et al*. (2017b) suggests that inoculation occurs during and immediately after birth via the vaginal canal, faeces, colostrum, skin, and saliva of the dam. However, Guzman *et al*. (2015) identified methanogens, fibrolytic bacteria and *proteobacteria* in the rumen of calves less than 20 minutes after birth, suggesting some inoculation may occur prior to birth. While there may be some microbes present prior to or at birth there are rapid changes in composition to reach a mature population (Jami *et al*. 2013). There is a complex eco system of ruminal microbes, the main types being: bacteria, archaea, protozoa, and fungi (Castillo-González *et al*. 2014; Wang *et al*. 2017). There are many types of bacteria found in the rumen (Castillo-González *et al*. 2014; Moon *et al*. 2021) and often function to break down specific carbohydrates (Russell *et al*. 2009; Castillo-González *et al*. 2014; Nagaraja and Titgemeyer 2007) or assist in the breakdown of lipids and proteins (Castillo-González *et al*. 2014). Archaeal species found in the rumen include anaerobic methanogens which use hydrogen as an energy source (Janssen and Kirs 2008). Ruminal microbes are anaerobic or facultatively anaerobic (Matthews *et al*. 2019) and the removal of hydrogen helps to support fermentation and favourable VFA production (Castillo-González *et al*. 2014; McAllister and Newbold 2008; Wolin 1979). Protozoa are key in preventing the risk of acidosis when highly fermentable feeds are consumed, due to their ability to ingest soluble sugars (van Zwieten *et al*. 2008). They also assist in the digestion of cellulose (a structural carbohydrate) (Yáñez-Ruiz *et al*. 2004). Ruminal fungi produce enzymes that assist in the digestion of cellulose and xylans (Castillo-González *et al*. 2014). Physiochemical properties of the rumen are key to support the symbiotic relationship with microbes. The temperature of the rumen is normally maintained at approximately 39 °C (Wahrmund *et al*. 2012), pH is normally maintained somewhere between 5.5 and 7.0 (Russell and Strobel 1989: Krause and Oeztel 2006), osmotic pressure of the rumen is normally approximately 250 mOsm/kg (Castillo-González *et al*. 2014) (mOsm/kg is a measure of osmolality and is the number of osmoles of solute per kilogram of solvent (Advanced renal education program n. d.).

Microbes throughout the entire GIT, including the intestines, gradually established with age and increase in abundance (Guo *et al*. 2020) with evidence suggesting that rumen microbiota reaches a mature state after around six months of age (Jami *et al*. 2013; Baldwin *et al*. 2004; Guo *et al*. 2020). However, the hindgut is believed to reach a stable microbial mature state earlier than the rumen (Guo *et al*. 2020). This is likely due to the development of the protective barrier function (Malmuthuge and Guan 2017), which prevents ruminal microbes affecting the intestinal populations (Guo *et al*. 2020). Within the intestines the rectum is believed to mature first due to its high luminal microbial growth rate (Guo *et al*. 2020). The duodenum has the lowest microbial growth rate (Guo *et al*. 2020). The presence of microbes in the hindgut provides an area for microbial digestion, aside from the rumen, with 11.6 to 17.0 % of total dietary cellulose, 2 to 11 % of total starch and 20 % of soluble carbohydrates undergoing microbial fermentation here (Ducharme *et al*. 2017). Alongside the fermentation function of the colon, it also functions to absorb water and controls the movement of digesta into the rectum (Ducharme *et al*. 2017).

The abomasum in mature ruminants continues to carry out acidic digestion, as it does in pre-ruminant animals (Ducharme *et al*. 2017). However, the relative size of the abomasum is smaller in mature ruminants, reducing in relative size from around twice as large as the rumen at birth to nine times smaller than the rumen by one year old (Fubini and Ducharme 2004). There are also age-dependent changes of the mucosal folds of the abomasum. The height and width of the folds increase as the animal ages with a peak at pubertal age, however, these decrease slowly after pubertal age (Hassan Omer *et al*. 2023). The secretion of pepsin is increased in mature ruminants (compared to at birth) to allow the animal to utilise protein from sources other than milk (Garnot *et al*. 1977).

In summary, there are many changes that occur throughout the entire gastrointestinal tract needed for it to reach a mature state, particularly in the rumen. The rumen must increase in capacity as well as develop papillae, muscular contractions and a microbial eco system in order to be functional. The abomasum and intestines do not appear to go through as many changes, however, there may be an increase in height and width of the folds in the abomasum and the intestinal microbial population reaches a mature state, likely occurring earlier than in the rumen.

2.3 Effect of preweaning management factors on the development of the gastrointestinal tract

Prior to weaning there are many management factors that need to be considered such as: colostrum, milk, milk replacer, milk allowance, feeding frequency, feeding method, solid feed provision, and additives. These factors and their potential influence on the GIT of the developing calf are discussed below.

2.3.1 Colostrum

Colostrum is the first mammary secretion produced following parturition (Playford and Weiser 2021). Colostrum is rich in fat, protein, vitamins, minerals (Mcgrath *et al*. 2016), amino acids (Blum and Hammon 2000), and bioactive compounds (e.g., oligosaccharides) relative to milk. Colostrum also contains bioactives including immunoglobulins, peptides, peptide hormones, growth factors, cytokines, lactoferrin, steroid hormones, triiodothyronine, thyroxine, nucleotides, polyamines, enzymes (Blum and Hammon 2000), and serum amyloid A3 (Molenaar *et al*. 2009). The concentration of these compounds, excluding lactose, decreases quickly over the first few days of lactation (Blum and Hammon 2000; Uruakpa *et al*. 2002) as transitional milk is produced and finally milk. A summary of the effect of colostrum on GIT development is provided in Table 2.1.

Table 2.1 Summary of the effect of colostrum on gastrointestinal tract development.

Trait	Effect[1]	Reference
LOWER GIT		
Small Intestinal mass	+	Shulman *et al*. (1990); Baumrucker *et al*. (1994)
Villus Height	+	Yang *et al*. (2015); Pyo *et al*. (2020); Van Soest *et al*. (2022)
Villus Width	+	Yang *et al*. (2015); Van Soest *et al*. (2022)
Crypt depth	+	Yang *et al*. (2015)
	=	Van Soest *et al*. (2022); Pyo *et al*. (2015)
Mucosal thickness	+	Yang *et al*. (2015); Van Soest *et al*. (2022)
VH/CP ratio	+	Yang *et al*. (2015); Van Soest (2022)
Microbiome population	+	Arshard *et al*. (2021); Malmuthage *et al*. (2015); Przybylska *et al*. (2007); Martin *et al*. (2021); Song *et al*. (2019)

[1]+: positive effect, =: no change.

Empirical work on colostrum suggests that bioactive compounds present in colostrum positively influence the development of the GIT and intestinal microbiota (Fischer *et al*. 2019), but this may be limited to the lower GIT. Due to the presence of oesophageal groove, limited amounts of colostrum enter the rumen, limiting its ability to directly affect ruminal development. Insulin, a key bioactive compound in colostrum, can increase the small intestinal mass (Shulman 1990) through the stimulation and proliferation of the intestinal epithelium (Baumrucker *et al*. 1994). Other bioactive compounds, lactoferrin, lysozyme, and lactoperoxidase, support the overall health of the GIT (Pakkanaen and Aalto 1997). The production of beneficial hormones, because of colostrum intake, indirectly stimulates intestinal maturation via circulation. Plasma glucagon-like peptide-1 (GLP1) and -2 concentrations increase after colostrum intake in calves fed colostrum soon after birth, compared to those that fed colostrum 12 hours after birth (Inabu *et al*. 2018). This increase aids in GIT development because GLP1 and -2 are important for glucose homeostasis (Fukumori *et al*. 2012) and stimulation of GIT growth (Taylor-Edwards *et al*. 2011).

Calves receiving colostrum, compared to those receiving bulk tank (saleable) milk have increased villus height (Yang *et al*. 2015; Pyo *et al*. 2020; Van Soest *et al*. 2022), villus width (Yang *et al*. 2015; Van Soest *et al*. 2022), crypt depth (Yang *et al*. 2015), mucosal thickness (Yang *et al*. 2015; Van Soest *et al*. 2022), and increased villus height to crypt depth ratio (Yang *et al*. 2015; Van Soest *et al*. 2022) in the small intestine. However, Van Soest *et al*. (2022) and Pyo *et al*. (2020) found no difference in crypt depth at three days old (Pyo *et al*. 2020) or five days old (Van Soest *et al*. 2022). Yang *et al*. (2015) concluded that calves receiving colostrum had a better-developed GIT with deeper, more uniform villi, and minimal cleaved tissues compared to calves receiving transition (between second and eighth milking after calving) or bulk tank milk. Colostrum can influence small intestinal development, and the sooner after birth that calves receive colostrum may result in improved development.

Colostrum also influences GIT development via the colonisation of bacteria (Arshard *et al*. 2021). Malmuthage *et al*. (2015) found that the intake of colostrum within the first 12 hours of life assisted in increased microbial colonisation of the small intestine, relative to calves that did not receive colostrum. *Lactobacillus* spp. and *Bifidobacterium* spp. are key bacteria which improve GIT development by proliferating mucous producing epithelial cells (Martin *et al*. 2021; Malmuthage *et al*. 2015; Song *et al*. 2019) and have protective functions of the intestines (Sanad *et al*. 2015). *Bifidobacterium* spp. also inhibit harmful *Escherichia coli (E. coli)* bacteria (Song *et al*. 2019). Malmuthage *et al*. (2015) also suggested that the timing of colostrum intake impacts microbial colonisation. The concentrations of bacterial species associated with the colon, such as *Bifidobacterium* and *Lactobacillus*, were increased in calves fed immediately with colostrum when compared to those fed 12-hours post birth. This suggests that the intake of colostrum as soon as possible after birth increases the rate of microbial colonisation (Malmuthage *et al*. 2015). Oligosaccharides, also found in

colostrum, can not only promote the growth of beneficial microorganism (Madhusoodanan 2022), but also assist in providing protection against pathogens through their action as competitive inhibitors that prevent the binding of pathogens to the intestinal epithelium (Przybylska *et al*. 2007; Petherick 2012), and thus, their establishment in the intestine. Colostrum can therefore influence the development of the GIT, by protecting against pathogens that may cause temporary or permanent damage to the GIT.

In summary, colostrum contains many bioactive compounds which aids the development of the GIT. A direct influence of colostrum intake on the rumen is limited due to the presence of the oesophageal groove. The published literature, however, demonstrates that colostrum directly and indirectly influences the development of the lower digestive tract. Nutrients and bioactives in colostrum elicit a direct effect on the proliferation of epithelium, secondly, they are absorbed into circulation and elicit their effect through circulatory and endocrine systems of the calf and lastly, they promote the growth of beneficial microorganisms for better protection and growth of the small intestine.

2.3.2 Milk versus milk replacer

Whole milk is the ideal feed for calves because it provides highly digestible nutrients and bio-actives to the calf during the first few months of life (Seegraber and Morrill 1986; Zabielski 1998). Milk replacers (MR) can be fed to calves as a substitute for whole milk and considered to be of good quality if they have a similar chemical composition to whole milk and has comparable proportions of digestible nutrients (Moran 2012). The advantage of MR over whole milk relates to cost and MR quality (Moran 2012) as MR can be formulated to provide consistent nutrients and additives to calves (Górka *et al*. 2021). Górka *et al*. (2018) proposed that a key difference between whole milk and MR is the presence of the bioactive compounds (Zalbielski 1998; Górka *et al*. 2011a), including butyric acid, a known stimulator of GIT development, in whole milk. Presence of numerous bioactive compounds in milk have been found to positively influence the development and establishment of beneficial microbiota in the intestine (Fischer *et al*. 2019). Processing steps such as homogenisation and drying, required to create MR powders, can damage bioactive components (Wilms *et al*. 2022) and this may negatively affect the GIT development in calves consuming poor quality MR. A summary of the effect of milk and MR is provided in Table 2.2.

Table 2.2 Summary of effect of milk and milk replacer (MR) on gastrointestinal tract development.

Trait	Management practice	Effect[1]	Reference
RUMEN			
Papillae Length	Milk	+	Górka *et al*. (2011a)
Papillae Width	Milk	+	Górka *et al*. (2011a)
LOWERGIT			
Microbiome population	Milk	+	Fischer *et al*. (2019)
Intestine	MR	-	Wood (2022); Seegraber and Morrill (1986); Blätter *et al*. (2001)

[1]+: positive effect, -: negative effect.

Milk replacer can be made from whole milk powder or manufactured using a skim milk, or whey-base (Moran 2012). Whey proteins are the primary component of MR and considered the optimal protein for calves (Grice *et al*. 2020). There are other replacers made from plant sources, but these are less favourable because they contain complex proteins and anti-nutritional factors that reduce digestibility in developing calves (Tanan 2005). For example, the use of poorly processed soy in MR's caused intestinal damage, lowered enzyme production, and decreased digestibility (Wood 2022). There is also evidence that MR slows intestinal development (Seegraber and Morrill 1986; Blättler *et al*. 2001), particularly when MR of plant sources are used (Seegraber and Morill 1986; Montagne *et al*. 1999; Drackley *et al*. 2006). Calves fed whole milk had a heavier jejunum, ileum, and total small intestine weight as well as larger papillae lengths and widths compared with calves receiving MR and MR supplemented with butyrate (Gòrka *et al*. 2011a). While this study provided evidence for lack of GIT development, because of MR intake, this may be due to the use of MR manufactured from soybean and the same result may not be seen with MR manufactured from other sources.

In summary, the effect of MR compared to whole milk on GIT development is unclear. While there is evidence that calves can efficiently digest and absorb quality MR within the first week of life (Liang *et al*. 2016), the quality of MR and its effect on GIT development can be variable. The effect is determined by its composition and by the ingredients and additives used in the formulation. The use of poor-quality protein and fat sources from food processing and oil extraction streams or over processing of the milk-based ingredients have the potential to negatively influence GIT development.

2.3.3 Milk allowance

Under natural conditions, the calf is left with the dam and will have many small feeds throughout the day (Muir 2022). This type of feeding is not viable under most artificial rearing systems due to labour costs associated with feeding and allowance is often restricted due to the consequential reduction in starter intake

with high milk allowances (Sweeney *et al*. 2010). The amount of milk provided to the calf is often dependent on the type of rearing system. For example, artificial rearing systems may allow calves to have *ad libitum* access to milk (Welboren *et al*. 2019), although, most conventional systems limit milk allowance to 10% of birth weight (Fischer *et al*. 2019). A summary of the effect of different milk allowance is provided in Table 2.3.

Table 2.3 Summary of effects of different milk allowances on gastrointestinal tract development.

Trait	Management practice	Effect[1]	Reference
OMASUM	Restricted	+	Geiger *et al*. (2016); Silper *et al*. (2014); Khan *et al*. (2007)
RUMEN	Restricted	+	Steele *et al*. (2016)
	Increased	+	Khan *et al*. (2007); Geiger *et al*. (2016)
Size	Restricted	+	Yohe *et al*. (2022)
		=	Silper *et al*. (2014)
	Increased	+	Khan *et al*. (2007); Geiger (2016)
Papillae length	Increased	+	Khan *et al*. (2007)
		=	Schäff *et al*. (2018)
Papillae width	Increased	+	Khan *et al*. (2007)
BHB concentration	Restricted	+	Jafari *et al*. (2020)
		=	Parson *et al*. (2021); Kazemi-Bonchenari *et al*. (2022)
	Increased	+	Khan *et al*. (2007)
Butyrate concentration	Increased	+	Khan *et al*. (2007)
Propionate proportion	Restricted	+	Yohe *et al*. (2022)
pH	Restricted	-	Yohe *et al*. (2022)
ABOMASUM	Restricted	+	Geiger *et al*. (2016); Khan *et al*. (2007)
LOWER GIT			
Propionate proportion	Restricted	+	Kumar *et al*. (2021)
Acetate proportion	Restricted	-	Kumar *et al*. (2021)
Small intestine length	Restricted	+	Kosiorowska *et al*. (2011); Yohe *et al*. (2022)
Microbial population	Increased	+	Kumar *et al*. (2021)
JEJUNUM			
Villi number	Restricted	+	Kosiorowska *et al*. (2011)
Crypt number	Restricted	+	Kosiorowska *et al*. (2011)
Crypt depth	Increased	-	Schäff *et al*. (2018)
SA of villi	Increased	+	Shäff *et al*. (2018)
VH:CP	Increased	+	Schäff *et al*. (2018)
Full weight	Restricted	=	Yohe *et al*. (2018)
DUODENUM	Increased	=	Schäff *et al*. (2018)
Full weight	Restricted	+	Yohe *et al*. (2022)
ILEUM	Increased	=	Schäff *et al*. (2018)
Muscle thickness	Restricted	+	Kosiorowska *et al*. (2011)
Full weight	Restricted	=	Yohe *et al*. (2022)
Caecum			
Full weight	Restricted	+	Yohe *et al*. (2022)

COLON			
Crypt depth	Restricted	+	Yohe *et al*. (2022)

[1]+: positive effect, -: negative effect, =: no difference.

There is contrasting evidence on the effect of milk allowance on forestomach development. Restricted milk allowance often encourages the intake of solid feed (Steele *et al*. 2016). This potential increase in solid feed intake stimulates rumen development due to the increased availability of substrates required to initiate development (Steele *et al*. 2016; Kertz and Loften 2013; Baldwin *et al*. 2004). More specifically, restricted milk allowance has resulted in increased size of the rumen (Yohe *et al*. 2022), increased proportions of rumen papillae (Schäff *et al*. 2018) and increased beta-Hydroxybutyric acid (BHB) concentrations (Jafari *et al*. 2020). BHB is an indicator for metabolic development of the rumen (Deelan *et al*. 2016). However, other studies have found contrasting evidence, with similar results found from higher milk allowances. Increased milk allowance has improved rumen development (Khan *et al*. 2007; Geiger *et al*. 2016) in terms of BHB and butyrate concentration after weaning (Khan *et al*. 2007), papillae length and width (Khan *et al*. 2007) and organ weight (Khan *et al*. 2007; Geiger *et al*. 2016). Omasum development (Geiger *et al*. 2016; Silper *et al*. 2014; Khan *et al*. 2007) and abomasum development (Geiger *et al*. 2016; Khan *et al*. 2007) were also improved in terms of organ weight when compared to more restricted milk allowances. Other studies have found even more contrasting evidence, concluding that milk allowance does not influence rumen development. Papillae length and papillae density were not different in calves fed an unrestricted allowance or restricted allowance (Scäff *et al*. 2018). Papillae size and empty reticulorumen weight also showed no difference between different restricted feeding levels (Silper *et al*. 2014). BHB concentrations were also found to be no different between different allowance levels (Parsons *et al*. 2021; Kazemi-Bonchenari *et al*. 2022). The contrasting evidence of the influence of milk allowance on forestomach development may be due to many different factors that were not consistent throughout the studies. Unrestricted milk allowance was compared to restricted allowances in Schäff *et al*. (2018), whereas other studies compared different, feeding levels (Silper *et al*. 2014; Geiger *et al*. 2016; Khan *et al*. 2007; Parsons *et al*. 2021; Kazemi-Bonchenari *et al*. 2022; Jafari *et al*. 2020). Solid feed was offered in all studies; however, some was offered at a restricted allowance (Geiger *et al*. 2016), while other studies offered it at *ad libitum* (Schäff *et al*. 2018; Silper *et al*. 2014; Khan *et al*. 2007a; Parsons *et al*. 2021; Kazemi-Bonchenari *et al*. 2022; Jafari *et al*. 2020). Weaning also occurred at different ages either around 60 days old (Schäff *et al*. 2018; Silper *et al*. 2014; Geiger *et al*. 2016; Jafari *et al*. 2020), at approximately 45 days old (Khan *et al*. 2007; Parsons *et al*. 2021) or at 53 days old (Kazemi-Bonchenari *et al*. 2022) and different weaning strategies were used. Some studies didn't specify weaning method (Geiger *et al*. 2016; Kazemi-Bonchenari *et al*. 2022), abrupt weaning was used by Silper *et al*. (2014) and step-down weaning was used by others (Schäff *et al*. 2018; Khan *et al*. 2007a; Parsons *et al*. 2021; Jafari *et al*. 2020). Measurements to assess GIT development were either taken at the time of weaning (Silper *et al*. 2014; Schäff *et al*. 2018; Geiger

et al. 2016), approximately two weeks after weaning (Khan *et al*. 2007a) or throughout the study (Parsons *et al*. 2021; Kazemi-Bonnchenari *et al*. 2022; Jafari *et al*. 2020). The inconsistencies between studies may be the cause for the contrasting evidence and make it difficult to conclude how milk allowance truly influences forestomach development. Overall, the empirical literature indicates that a higher milk allowance negatively influences the solid feed intake and therefore rumen development in calves younger than two months of age. The use of gradual or step-down weaning could encourage solid feed intake and fast tract rumen development to mitigate the negative effects of high allowances (Khan *et al*. 2016).

Similar to the forestomach, there is contrasting evidence on the effects of milk allowance on lower GIT development. Kosiorowska *et al*. (2011) found an increase in small intestinal length for calves on lower milk allowances as well as an increase in villi and crypt number in the jejunum. Schäff *et al*. (2018) also found increased development in the jejunum with unrestricted milk allowance resulting in greater villus circumference in the mid jejunum and greater cut surface area of villi, greater villus height/ crypt depth ratio and smaller crypt depth in the distal jejunum. However, Schäff *et al*. (2018) found no difference in the duodenum or ileum, but Kosiorowska *et al*. (2011) found an increase muscle thickness in the ileum of calves on a lower milk allowance. Despite these studies finding no effect of allowance on duodenum development, Yohe *et al*. (2022) found evidence for increased duodenum development (full weight (kg), full weight (% body weight (BW)) and empty weight (%BW) on lower milk allowances compared to higher milk allowances, but no difference in other areas of the small intestine. In the large intestines, however, the caecum has increased full weight (kg and %BW) and the colon had increased crypt width for low milk allowances compared to a higher milk allowance (Yohe *et al*. 2022).

Again, the contrasting evidence may be a result of contrasting factors between studies. All studies provided unrestricted solid feed. Yohe *et al*. (2022) and Kosiorowska *et al*. (2011) compared two different milk allowance levels whereas, Schäff *et al*. (2018) compared unrestricted milk allowance to a restricted level. The measurement to assess GIT development occurred at weaning, using a step-down method, for Schäff *et al*. (2018) and Yohe *et al*. (2022) at around 60 days old. Kosiorowska *et al*. (2011) did not distinguish weaning method, but assessment was carried out at 36 days as well as at weaning at 56 days old.

Milk allowance may also influence the microbiome of the GIT. While total short-chain fatty acid (SCFA) concentrations were not different between two milk allowance treatments (Kumar *et al*. 2021), in the rumen, lower milk allowances resulted in increased proportion of propionate as well as decreased pH (Yohe *et al*. 2022). In the lower GIT, there is conflicting evidence. Increased allowances decreased the acetate and increased the propionate proportion (Kumar *et al*. 2021). Whereas Yohe *et al*. (2022) found a decreased faecal proportion of acetate for calves on lower milk allowances. Changes in pH may elicit an effect of the GIT

microbiome due to certain microbes requiring a particular pH to thrive (Jin and Kirk 2018). This may be indirectly influenced by VFAs, as changes in proportion of these may induce changes in pH (Ramos *et al*. 2021). Specifically, the bacterial population in the lower GIT appears to be influenced by allowance. Calves on *ad libitum* diets have a greater bacterial diversity with *Faecalibacterium* being three times higher in *ad libitum* calves compared to calves on an allowance of 10% body weight (Kumar *et al*. 2021).

In summary, milk allowance has an indirect effect on rumen development; restricted allowances result in increased solid feed consumption which stimulates rumen development. However, other studies have found that increased allowances have had the same result. This contrast in evidence may be related to inconsistencies between studies such as offer of solid feed, treatment levels, weaning method and time of measurement. There is also conflicting evidence in terms of lower GIT development as a result of different milk allowances. Restricted milk allowances resulted in increased number of villi and crypts. Whereas increased milk allowances resulted in larger villi and smaller crypt depth in the jejunum. Empirical studies suggest that the duodenum may not be affected by milk allowance. There is some evidence that the GIT microbiome may be influenced by milk allowance, particularly the bacterial population.

2.3.4 Feeding frequency and feeding method.

A summary of the effect of feeding frequency and feeding method of GIT development is provided in Table 2.4.

Table 2.4 Summary of effects of different feeding frequency and feeding methods on gastrointestinal tract development.

Trait	Management practice[2]	Effect[1]	Reference
Saliva production	Artificial Teat	+	Moran (2012)
RUMEN			
Papillae length	Increased FF	=	Kehoe *et al*. (2007)
Papillae width	Increased FF	=	Kehoe *et al*. (2007)
Wall thickness	Increased FF	=	Kehoe *et al*. (2007)
Capacity	Increased FF	+	Mirzaei-Alamouti *et al*. (2020)
Total VFA concentration	Decreased FF	+	Jafari *et al*. (2021)
		=	Ahmadi *et al*. (2022)
Proportion of butyrate	Decreased FF	+	Jafari *et al*. (2021)
Proportions of VFAs	Decreased FF	=	Ahmadi *et al*. (2022)
BHB concentration	Decreased FF	=	Ahmadi *et al*. (2022); Grice *et al*. (2022)
pH	Decreased FF	-	Mirzaei-Alamouti *et al*. (2020)
		=	Ahmadi *et al*. (2022)
	Artificial Teat	+	Araujo-Febres and Vergara López (2007);

			Palma-Hidalgo *et al*. (2021)
Microbial population	Artificial Teat	+	Beede (2012)
LOWER GIT			
Microbial population	Increased FF	=	Zhang *et al*. (2021)

[1]+: positive effect, -:negative effect, =: no difference.

[2]FF= feeding frequency.

2.3.4.1 Feeding frequency

Young calves left with their mother suckle them multiple times a day (e.g., cow-calf beef system) and the frequency of suckling is generally reducing as the calf ages (Jafari *et al*. 2021). In the artificial rearing of calves, milk is generally offered once or twice daily (Moran 2012) and therefore may hinder or restrict the expression of natural suckling behaviour of young calves. Availability and adaptation of automatic feeding systems, on commercial farms, could allow an opportunity to increase milk feeding frequency that resembles the natural behaviour of calves (Jafari *et al*. 2021; Jensen and Holm 2003). Often, changes in feeding frequency coincide with differences in milk allowance which has been discussed in previous sections.

Research has demonstrated that structural development of the rumen is not influenced by changes in milk feeding frequency. No differences in rumen papillae length, width, and rumen wall thickness were reported when calves were offered the same amount of milk once or twice daily (Kehoe *et al*. 2007). However, rumen capacity may be greater in once-a-day feeding compared to three-times-a-day feeding, implied by the increased grazing time of the once-a-day group (Mirzaei-Alamouti *et al*. 2020). Initiation of rumen fermentation results in the production of VFA's, and their concentration in the rumen can be used as an indicator for rumen development (Graham and Simon 2005). Total ruminal VFA and molar proportion of butyrate was greater in twice- versus three-times-a-day frequencies (Jafari *et al*. 2021). However, Jafari *et al*. (2021) proposed that this observed increase in rumen VFA concentration in twice daily fed calves may be due to increased starter intake, leading to a consequent increase in rumen fermentation. Ahmadi *et al*. (2022) study contradicted this, finding no difference in total VFA and molar proportions of VFAs. This was also supported by a lack of differences in BHB concentrations (Ahmadi *et al*. 2022; Grice *et al*. 2022). A decline rumen pH and an increase in time spent ruminating also indicates rumen fermentation and development (DeVries *et al*. 2009; Mirzaei *et al*. 2015). However, there is conflicting evidence; ruminal pH was lower for once-daily feeding compared to three-time-a-day (Mirzaei-Alamouti *et al*. 2020), but showed no difference when twice-a-day feeding was compared to three-times-a-day (Ahmadi *et al*. 2022). In conclusion, the feeding frequency of milk has minimal effect on the development of the rumen.

The abomasum is an important area during the milk feeding phase as it is the key area for digestion (Guillteau *et al*. 2009a). Feeding frequency may influence abomasal emptying (Aguera Pinheiro *et al*. 2020), the time

digesta remains in the abomasum before being passed into the intestines (Burgstaller *et al*. 2017). Prolonged abomasal emptying is associated with increased GIT disease, particularly bloat (Burgstaller *et al*., 2017), which can be deadly, due to increased fermentation time (Aguera Pinheiro *et al*. 2020). Increased milk feeding is a proposed method to reduce the occurrence of bloat (Burgstaler *et al*. 2017) due to an increase in abomasal emptying (Orellana Rivas *et al*. 2022). The effect of feeding frequency on abomasal emptying may have some influence on intestinal development as digesta enters the intestines next. However, there is little literature describing the potential influence of this. It appears that overall, there is little literature regarding feeding frequency and lower GIT development in calves. One study has shown that microbial composition was unchanged in the ileal and colonic digesta and colon mucosa across three different feeding levels (one, three or five feeds a day) in pigs (Zhang *et al*. 2021), whether this translates to calves remains to be determined. In conclusion, abomasal emptying may be increased by increased milk feeding frequency. The influence of feeding frequency on intestinal development is unknown.

2.3.4.2 Feeding method

Farmers use a variety of methods to deliver milk to calves. Most commonly, calves are fed from buckets (Moran 2012; Horvath and Miller-Cushon 2017), troughs (Moran 2012) or using teat feeders (Moran 2012; Horvath and Miller-Cushon 2017), such as calfaterias. Teats help calves elicit natural suckling behaviours (Horvath and Miller-Cushon 2017). Milk delivery methods used may interact with feeding frequency to influence feed intake and thereby influence the development of the digestive tract, however, elucidating this aspect is scarce.

Feeding milk through an artificial teat compared to bucket feeding was found to be beneficial for increasing saliva production (Moran 2012). Increased saliva production can aid rumen function due to increased fluid flow, which helps maintain fluidity of the rumen (Palma-Hidalgo *et al*. 2021), support ruminal microbes (Beede 2012), and improve rumen buffering capacity (Palma-Hidalgo *et al*. 2021). Increased saliva production provides sodium, potassium, bicarbonate, and phosphate which helps to maintain the rumen pH within its ideal range of pH 5.5 to pH 7.0 (Araujo-Febres and Vergara López 2007; Russell and Strobel 1989; Krause and Oeztel 2006).

The development of other areas of the GIT may also be influenced by the methods used to feed milk. Bucket and trough feeders are required to be at least 30cm off the ground, from where the calf stands, to ensure the correct function of the oesophageal groove (Moran 2012). Dysfunction, or poor development, of the groove, can result in milk entering the rumen and causing significant health implications due to acidosis (Kaba *et al*. 2018). In summary, there is some evidence that teat feeding may help aid rumen function and bucket and

trough feeding have the potential to cause oesophageal groove dysfunction, but evidence of differences in rumen and GIT development based on the method of milk feeding is lacking and not conclusive.

Overall, there is a lack of evidence on how feeding frequency and feeding method can affect the development of the GIT. There is conflicting evidence as to the effect of feeding frequency on rumen development but there appears to be little effect, with any differences being attributed to other factors. There is even less evidence for the effect of frequency on the lower GIT, however there is potential that the effect of frequency on abomasal emptying may have a flow-on effect to the intestine. The effect of feeding method on development is unclear and differences between feeding methods are more related to supporting function rather than development.

2.3.5 Solid feed

Solid feeds are typically provided to artificially reared calves before weaning and can include concentrates, forages (grass/hay) or a combination of the two. The type and quantity of solid feed provided to calves are often dependent on the type of rearing system (Cozzi *et al*. 2002), cost (Blanco *et al*. 2008), and milk allowance (Sweeny *et al*. 2010). Calves start eating a significant amount of solid feed during the third week of their age (Khan *et al*. 2011a). This initiates the rumen fermentation (Baldwin *et al*. 2004), and fermentation end products and along with feed bulk triggers the rumen development (Khan *et al*. 2016). The provision of solid feeds has been shown to promote both structural and metabolic development of the rumen (Steele *et al*. 2016) and initiate differentiation of epithelium into papillae (Kertz and Loften 2013; Baldwin *et al*. 2004). A summary of the effects of solid feed is provided in Table 2.5.

Table 2.5 Summary of effects of different solid feeds on gastrointestinal tract development.

Trait	Management practice[2]	Effect[1]	Reference
Forestomach weight	Corn- and wheat-based starter	+	Khan *et al*. (2008)
Saliva production	C	-	Chibisa *et al*. (2016); Maekewa *et al*. (2002)
RUMEN	R	+	Steele *et al*. (2016)
Weight	F	+	Pazoki *et al*. (2017); Castells *et al*. (2013)
Fermentation	R	+	Baldwin *et al*. (2004)
Papillae development	R	+	Kertz and Loften (2013); Baldwin *et al*. (2004); Khan *et al*. (2008)
Papillae length	C	-	Diao *et al*. (2019)
VFA production	C	+	Poier *et al*. (2022); Heinrichs and Lesmeister (2004);

			Mentschel *et al*. (2001)
Muscular development	F	+	Beiranvand *et al*. (2014); Mirzaei *et al*. (2015)
Microbial population	C	D	Yáñez-Ruiz *et al*. (2010)
	R	D	Guzman *et al*. (2016)
pH	F	+	Poier *et al*. (2019); Khan *et al*. (2011b); Castells *et al*. (2012)
LOWER GIT			
Small intestinal length	R	+	Choudary *et al*. (2021)
Large intestinal length	R	+	Choudary *et al*. (2021)
Jejunum villus length	R	=	Choudary *et al*. (2021)
Jejunum crypt depth	R	=	Choudary *et al*. (2021)
Colon crypt depth	R	=	Choudary *et al*. (2021)
Microbial population	F	+	Knudsen *et al*. (2012); Van Hees *et al*. (2019)
	R	=	Choudary *et al*. (2021)

[1]+: positive effect, -:negative effect, =: no difference, D: difference seen (positive or negative effect not specified.

[2] R= receiving solid feed (type not specified), C= concentrate, F= forage.

Concentrate diets contain high amounts of energy (Poier *et al*. 2022), due to readily fermentable carbohydrates (Khan *et al*. 2016). They are also highly palatable (Khan *et al*. 2016), easily digestible (Poier *et al*. 2022) and commonly provided to calves. Physical form and ingredients of concentrate diets influences the intake and rumen fermentation profile. For example, corn- and wheat-based diets have resulted in increased weight of the forestomach and papillae growth when compared to barley- and oat- based diets (Khan *et al*. 2008). Concentrate diet result in increased VFA production, compared to forages (Poier *et al*. 2022; Heinrichs and Lesmeister 2004; Mentschel *et al*. 2001). A key VFA, butyrate, triggers epithelial development (Baldwin *et al*. 2004) and papillae development (Castells *et al*. 2012; Heinrichs and Lesmeister 2004). It provides energy for the thickening of rumen wall, papillae growth, and triggers capillary development (Weignard *et al*. 1975). Propionate is less influential on rumen development but may increase thickening of the stratum corneum in both lambs (Rickard and Ternouth 2009) and calves (Gilliand *et al*. 1962), although this may be due to a combined effect with butyrate. Studies have shown that forage feeds can have a positive influence on structural rumen development. Inclusion of forage has increased ruminal growth (McLeod and Baldwin 2000), with an increased empty rumen weight for calves supplemented with hay (Pazoki *et al*. 2017; Castells *et al*. 2013), likely via improved muscular development (Beiranvand *et al*. 2014; Mirzaei *et al*. 2015) and stimulation of morphological development of the epithelial cells (Diao *et al*. 2019).

Increased production of VFAs can cause a decrease in ruminal pH (Ramos *et al*. 2021). This can affect the rumen microbial population (Penner and Oba 2009), as microbes require a particular pH to thrive (Jin and Kirk 2018). Low ruminal pH reduces the population of cellulolytic bacterial population (Franzolin and Dehority 1996), and the establishment of ciliate protozoa (Eadie 1962). This was supported by a study finding that lambs with access to grass hay and concentrates had different ruminal bacteria populations, compared to those with access to only grass hay (Yáñez-Ruiz *et al*. 2010). These differences persisted for at least for 4-months post-weaning, even when post-weaning diets were the same. Although this study involved lambs, the effect of feed-type would likely have a similar influence on a calf's ruminal bacteria population. Calves also had different microbial populations throughout the GIT when they received only milk versus milk and solid feed (Guzman *et al*. 2016). This persistent change in microbial population could be related to changes in ruminal pH. High concentrate diets result in decreased saliva production (Chibisa *et al*. 2016), compared to forage diets (Maekewa *et al*. 2002). This can have significant implications on rumen pH due to the buffering function of saliva (Palma-Hidalgo *et al*. 2021)

Changes in physical form of feed, such as particle size, have been found to affect papillae size. Calves consuming a ground diet had shorter papillae, with a reduced surface area compared to unground diets (Diao *et al*. 2019). Concentrate diets typically have a smaller particle size than forages and this can have further implications on rumen pH (Kmicikewycz and Heinrichs 2015; Beauchemin *et al*. 2003). In contrast, inclusion of hay in the diet increased ruminal and faecal pH compared to concentrates (Poier *et al*. 2019) and stabilised ruminal pH (Khan *et al*. 2011b; Castells *et al*. 2012).

In conclusion, solid feeds are important for GIT development as they provide a substrate to initiate rumen development (Steele *et al*. 2016; Ketrz and Loften 2013; Baldwin *et al*. 2004). Concentrate feeds appear to be more beneficial due to the increased production of butyrate, however, there the risk of negative influence on ruminal pH. The negative effect on ruminal pH may be mitigated by the provision of forages, particularly hay, which help stabilise the rumen. Therefore, it may be most beneficial to provide a combination of concentrates and forages to the developing calf.

The lower GIT may also be influenced by solid feed intake, however, published literature is scarce. It was reported that the butyrate that reaches the intestines can help stimulate epithelial proliferation (Górka *et al*. 2011b; Kowalski *et al*. 2015), in aid of the increased butyrogenesis. Much of the intestinal growth and maturation occurs throughout the first week of life (Poier *et al*. 2022). The increased butyrogensis likely stimulates this intestinal growth and maturation (Górka *et al*. 2011a; Steele *et al*. 2016). Small intestinal length, large intestinal length, and total intestinal length was greater for piglets who had access to solid feeds (Choudary *et al*. 2021). However, there were no significant differences in terms of villus length in the jejunum

or crypt depth in the jejunum or colon between piglets receiving milk and piglets receiving milk and solid feed (Choudary *et al*. 2021). It is likely calves would have the same trend as their lower GIT is relatively analogous to that of a monogastric animal (Gäbel *et al*. 2002; Steele *et al*. 2016; Kaba *et al*. 2018). The provision of more fibrous feeds, such as forage, may be beneficial in large intestinal development (Knudsen *et al*. 2012; Van Hees *et al*. 2019) as fibrous feeds pass through the small intestine and reach the large intestine, providing a substrate for microbial fermentation and VFA production (Choudary *et al*. 2021). Solid feed provision, comparing piglets on a milk versus milk and solid diet showed no impact on microbial population in the small intestine but had significant differences in the colon (Choudary *et al*. 2021) and the colon microbiome population was significantly different to other areas of the intestine (Choudary *et al*. 2021; Zhao *et al*. 2015; Mu *et al*. 2017; Crespo-Piazuelo *et al*. 2018; Holman *et al*. 2017). However, these suggestions came from studies on pigs and this effect may be limited in calves due to the action of the rumen. The differences in microbes in certain areas in the GIT may be related to pH. The pH of digesta in the caecum and colon was decreased by the provision of concentrate feeds, compared to milk only, but remained unchanged in the small intestine (Choudary *et al*. 2021). This may alter microbial populations due to some microbes requiring a particular pH to survive (Jin and Kirk 2018).

In summary, solid feed appears to influence the intestine differently to liquid feed specifically when considering their length and microbiome population. Morphological development, pH and microbiome population of the rumen seems to be influenced by provision of solid feed. There is a need for more research around the effect of solid feed on the development of the lower GIT, specifically relating to calves or research comparing different types of solid feeds.

2.3.6 Additives

Feed additives are defined by the Ministry for Primary Industries (MPI) (n.d.) as “non-nutrient ingredients... added to feed to improve its quality, taste, or nutritive value.” Common feed additives used in calf nutrition are probiotics, prebiotics, probiotic and prebiotic mixtures (synbiotics), butyrate and nucleotides. There are potential limitations to the use of additives if supplemented in milk or MR as the action of the oesophageal groove will limit the amount entering the rumen, therefore limiting the ability to influence rumen development (Baldwin *et al*. 2004). A summary of the effects of different additives and their administration method on gastrointestinal development is provided in Table 2.6.

Table 2.6 Summary of the effects of different feed additives on gastrointestinal tract development.

Trait	Management practice	Administration Route[2]	Effect[1]	Reference
RUMEN				
Microbial population	Probiotics	LF	+	Zhang *et al*. (2019)
		MA	+	Chaucheyras-Durand and Fonty (2002)
		ND	+	Cangiano *et al*. (2020)
	Butyrate		+	Rodgríguez *et al*. (2022)
Papillae length	Probiotics	ND	+	Xiao *et al*. (2016)
		SF	+	Lesmeister *et al*. (2004)
	Prebiotics	LF	+	Arne and Ilgaze (2021); Alves Costa *et al*. (2019)
	Synbiotics	ND	+	Xiao *et al*. (2016)
		LF	+	Brewer *et al*. (2014)
	Butyrate	LF	+	Górka *et al*. (2011b)
		SF	+	Górka *et al*. (2011b)
Papillae width	Probiotics	SF	+	Lesmeister *et al*. (2004)
	Prebiotics	LF	+	Arne and Ilgaze (2021)
Weight	Probiotics		+	Harris *et al*. (2017)
Butyrate concentration	Probiotics	ND	+	Xiao *et al*. (2016)
Wall thickness	Probiotics	SF	+	Lesmeister *et al*. (2004)
	Prebiotics	LF	+	Arne and Ilgaze (2021)
pH	Probiotics	ND	=	Xiao *et al*. (2016)
			D	Seo *et al*. (2010); Cangiano *et al*. (2010)
VFA concentration	Probiotics	ND	=	Xiao *et al*. (2016)
			D	Seo *et al*. (2010); Cangiano *et al*. (2010)
	Prebiotic	ND	+	Chang *et al*. (2022)
Fermentation	Prebiotics	ND	+	Chang *et al*. (2022)
LOWERGIT	Nucleotides	LF	=	Kehoe *et al*. (2008)
Microbial population	Probiotics	LF	+	Timmerman *et al*. (2015); Liu *et al*. (2022)
		ND	+	Uyeno *et al*. (2015); Isolauri *et al*. 2001
	Prebiotics	ND	+	Cangiano *et al*. (2020); Spring *et al*. (2015)
		LF	+	Uyeno *et al*. (2013)
	Synbiotics	LF	+	Geigerová *et al*. (2017)
		SF	+	Kridtayopas *et al*. (2019)
	Nucleotides	LF	-	Górka *et al*. (2021)
			+	Król (2011)
	Amino acids	SF	+	Hou *et al*. (2023)

SMALL INTESTINE				
Mucosal thickness Duodenum	Probiotics	MA	+	Wu *et al*. (2022)
Villus height	Probiotics	MA	+	Wu *et al*. (2022)
	Prebiotics	ND	+	Uyeno *et al*. (2015)
		LF	+	Castro *et al*. (2016)
	Synbiotics	SF	+	Kridtayopas *et al*. (2019)
	Butyrate	LF	+	Guilloteau *et al*. (2009b)
Crypt depth	Probiotics	MA	+	Wu *et al*. (2022)
VH:CD	Probiotics	MA	-	Wu *et al*. (2022)
	Synbiotics	SF	+	Kridtayopas *et al*. (2019)
JEJUNUM	Probiotics	MA	+	Wu *et al*. (2022)
	Prebiotics	LF	-	Masanetz *et al*. (2010)
Villus height	Probiotics	MA	+	Wu *et al*. (2022)
	Butyrate	LF	+	Guilloteau *et al*. (2009b)
	Nucleotides	MA	+	Dinardo *et al*. (2022)
Crypt depth	Probiotics	MA	+	Wu *et al*. (2022)
	Nucleotides	MA	+	Dinardo *et al*. (2022)
ILEUM				
Villus height	Probiotics	MA	-	Wu *et al*. (2022)
	Synbiotics		+	Kridtayopas *et al*. (2019)
Crypt depth	Probiotics	MA	-	Wu *et al*. (2022)
	Synbiotics	SF	+	Kridtayopas *et al*. (2019)
COLON				
Crypt depth	Prebiotics	LF	+	Castro *et al*. (2016)

[1]+: positive effect, -:negative effect, =: no difference, D: difference seen (positive of negative effect not defined).

[2]LF= liquid feed, SF= solid feed, MA= manual administration, ND= not distinguished.

2.3.6.1 Probiotics

Probiotics are described as microorganisms that can be consumed and benefit the host (Várhidi *et al*. 2022). Probiotics have been found to promote rumen function in adult ruminants (Diao *et al*. 2019). However, Chaucheyras-Durand and Durand. (2010) suggested that probiotics may be limited in their ability to influence the rumen development in young ruminants. Most of the prebiotic and probiotic research has been focused on the health and development of the intestine.

Microbial populations appear to be influenced by the supplementation of probiotics. Supplementation of *Lactobacillus rhamnosus* (probiotic) for 6-weeks before weaning improved rumen bacteria diversity and microbial protein concentrations while increasing rumen VFA production (Zhang *et al*. 2019). Yeast, another probiotic, had similar results in the rumen. It promoted microbial colonisation, helped in the establishment

of rumen fermentation in lambs (Chaucheyras-Durand and Fonty 2002), improved the maintenance of microbial balance in the rumen, and increased microbial activity (Cangiano *et al*. 2020). It may also alter the concentrations of bacteria in the rumen (Cangiano *et al*. 2020), resulting in increased butyrate production (Xiao *et al*. 2016), and increased papillae length (Xiao *et al*. 2016). Yeast's assistance in the structural development of the rumen was supported by Lesmeister *et al*. (2004), who found that supplementation of 2% yeast culture increased papillae length and width and rumen wall thickness at weaning (5 weeks) (Lesmeister *et al*. 2004). There is conflicting evidence around the effect of probiotics on ruminal pH and VFA production. Xiao *et al*. (2016) concluded that ruminal pH or VFA concentrations are not altered by yeast supplementation However, Seo *et al*. (2010) suggested that probiotics help to prevent lactate production, in mature ruminants, which helps to maintain normal rumen fermentation patterns (therefore VFA production and pH), and Cangiano *et al*. (2020) suggested that supplementation with yeast stabilised ruminal pH and is beneficial for cellulolytic bacteria colonisation.

Probiotics can benefit intestinal microbiota (Timmerman *et al*. 2015). Lactic acid bacteria (LAB) and *Bacillus* species generally target the lower GIT (Uyeno *et al*. 2015), help to stabilise the microbiome (Uyeno *et al*. 2015; Liu *et al*. 2022), and decrease the risk of pathogen colonisation (Isolauri *et al*. 2001). Yeast also helped to regulate the microbiome (Liu *et al*. 2022). Pathogens use sources of carbon and nitrogen derived from microbiota for their growth and can change GIT conditions (Bäumler and Sperandio 2016), which can be harmful to beneficial microbes. Alpha diversity (species diversity within a community (Andermann *et al*. 2022)) of microbes was highest for calves receiving a combination of LAB and yeast compared to calves receiving LAB and yeast individually or no probiotics (Liu *et al*. 2022). Structural development may also be improved via the use of probiotics. Supplementation with a multispecies probiotic increased villus height in the duodenum and jejunum, increased crypt depth in the duodenum and increased mucosal thickness in all areas of the small intestine compared to calves challenged with *Escherichi coli* (Wu *et al*. 2022). However, the effect of probiotics may not affect all areas the same, as control calves had increased villus height and crypt depth in the ileum and increased villus height to crypt depth ratio in the duodenum and jejunum compared to supplemented calves (Wu *et al*. 2022).

In summary, probiotics are beneficial in stabilising the microbiome of the rumen and intestine. They also improved structural aspects of the rumen such as papillae length, papillae width and rumen weight. This may be a result of increased bacterial populations resulting in increased butyrate production. The effect of probiotics on ruminal pH and VFA concentrations is unclear. Structural development of the duodenum and jejunum is improved with probiotic supplementation; however, the effect may not be the same in the ileum.

2.3.6.2 Prebiotics

Prebiotics, such as specific oligosaccharides, are not digested by the host's digestive enzymes (Arne and Ilgaze 2021), but instead are broken down and utilised (Chang *et al*. 2022) by ruminal and intestinal microbes. Supplementation with different types of prebiotics can alter rumen epithelial development (Ghosh and Mehla 2012; Costa *et al*. 2019; Lopes *et al*. 2021). Mannan oligosaccharides (MOS) increase papillae development (Arne and Ilgaze 2021), specifically increasing papillae length (Alves Costa *et al*. 2019; Arne and Ilgaze 2021) and width (Arne and Ilgaze 2021). Galactooligosaccharides (GOS) were found to promote rumen fermentation (Chang *et al*. 2022). Total VFA concentrations, alongside acetate and propionate, were increased when calves were supplemented with GOS (Chang *et al*. 2022). Rumen VFA production may be used as an indicator for rumen development (Graham and Simon 2005), however, an increased build-up of VFA could reduce rumen pH and thereby negatively influence rumen microbial populations and fermentation (Penner and Oba 2009; Jin and Kirk 2018).

Prebiotics can benefit the structural development of the intestine, however, due to their digestion by rumen microbes (Chang *et al*. 2022), they will not reach the lower digestive tract unless protected or supplemented in milk or MR (Uyeno *et al*. 2015). Villus height was increased when either MOS (Cangiano *et al*. 2020) or GOS (Castro *et al*. 2016) were added to the diet. It was proposed that the increase in villus height, because of MOS, was due to the increase in the substrate for MOS-utilising bacteria which in turn elicits an increase in villus height (Cangiano *et al*. 2020). This increase in substrate also supports the MOS-utilising bacteria population (Cangiano *et al*. 2020). GOS supplementation has also been found to increase colonic crypt depth (Castro *et al*. 2016). Microbial population is supported by prebiotics as they competitively bind pathogenic bacteria, in hindgut fermenters and monogastric animals (Spring *et al*. 2015), and cello-oligosaccharides (CO) further supports microbiome as it is the fermentation substrate for cellulolytic bacteria (Uyeno *et al*. 2013). However, the influence of prebiotics on the lower GIT may be very type specific, not all prebiotics will have the same result. For example, supplementation with inulin resulted in decreased jejunal villus height and reduced proliferation in the ileum (Masanetz *et al*. 2010).

In summary, prebiotics increase epithelial development in the rumen, improving papillae growth. Ruminal fermentation also appears to be improved by prebiotic supplementation. Prebiotics can influence lower GIT development if protected or supplemented in liquid feeds. Influence of MOS and GOS improved villus height. GOS also improved crypt depth in the colon. Prebiotics may also benefit the microbiome of the intestines via the provision of substrates and prevention of pathogen binding.

2.3.6.3 Synbiotics

Synbiotics are a combination of prebiotics and probiotics (Schrezenmeir and de Vrese 2001). Papillae length and width were increased when calves were supplemented with inulin (prebiotic) in combination with *Escherichia faecium* (probiotic) and when fermentation end products of yeast (probiotic) in combination with glycan and oligosaccharides (prebiotics) were used (Brewer *et al*. 2014; Xiao *et al*. 2016). The inulin and *E. faecium* combination also resulted in increased muscle layer thickness (Arne and Ilgaze 2021).

Literature surrounding the effect of synbiotics on intestines, specific to calves, is scarce. However, a combination of fructooligsaccharides (FOS), GOS, and inulin improved the activity and survival of bacterial population in the intestine of calves (Geigerová *et al*. 2017). Recent research in chickens has shown improved structural development of the intestine (increased villus height/ crypt depth ratio and goblet cell number) in the duodenum and ileum of calves receiving a synbiotic (Kridtayopas *et al*. 2019). The microbiome was also improved; higher concentration of *bacillus sp.* in the small intestine and caecum, increased *Lactobacillus sp.* concentration in the jejunum, ileum, and caecum along with decreased *E. coli* concentration in the jejunum and caecum (Kridtayopas *et al*. 2019). This was in comparison to chickens receiving only a prebiotic. While this study was on a monogastric animal, it can be expected that similar results may be seen in calves as the lower GIT is relatively analogous to that of a monogastric (Gäbel *et al*. 2002; Steele *et al*. 2016; Kaba *et al*. 2018). Overall, they appear to have a positive effect on the structural development of the rumen and microbiome of the intestine. They may also have some positive influence on the structural development of the intestines.

2.3.6.4 Butyrate

Butyrate is another key additive, supplemented in MR/milk or solid feed (Górka *et al*. 2018). It is key for overall GIT development. When supplemented in solid feed, butyrate can influence rumen development and function (Górka *et al*. 2018). When sodium butyrate was added to the diet, development of the papillae increases (Górka *et al*. 2011b) and ruminal bacteria counts increased (Rodgríguez *et al*. 2022). When butyrate is supplemented in milk or MR it can elicit effects on the abomasum and small intestine (Górka *et al*. 2018). Villus height, and tunica mucosa thickness were increased in the duodenum and jejunum via butyrate supplementation, however, there was no difference found in the middle section of the jejunum (Guilloteau *et al*. 2009b). Supplementation may also improve the digestibility of nutrients in calves due to improved secretions of digestive enzymes. Sections of chymotrypsin, lipase, and pancreatic juice were increased (Guilloteau *et al*. 2010) along with increased activity of the main brush border enzymes (Guilloteau *et al*. 2009b; Górka *et al*. 2011a, 2014).

2.3.6.5 Nucleotides

Nucleotides, including DNA nucleotides (Dinardo *et al*. 2022), can be added to the diet. They are thought to promote cell division (Daneshmaud *et al*. 2017) and therefore result in increased GIT development. The use of nucleotides in calf diets has resulted in increased duodenal villus length and crypt depth (Dinardo *et al*. 2022). In contrast, Kehoe *et al*. (2008) found evidence that nucleotide supplementation did not influence structural development of the intestine, with no difference being found when compared to a probiotic supplementation group or a no supplementation group. Also, the supplementation of nucleotides decreased *lactobacillus sp.* in faeces of calves (Górka *et al*. 2021). Nucleotides may have a negative effect on some lower GIT microbes. Literature on the influence of nucleotides on rumen development is very scarce but yeast nucleotides may be beneficial in increasing total bacteria counts in rumen fluid (Król 2011). In summary, research surrounding the influence of nucleotides on the structural development of the intestines is conflicting and may have ambiguous effects on microbial populations in the intestine. There is little research on how nucleotides effect rumen development.

2.3.6.6 Amino acids

Amino acid supplementation may be important for calves receiving MR as its formulation may not meet amino acid requirement of the calf (Silva *et al*. 2021), this is particularly important when poor quality MRs are used. However, research regarding amino acids influence on GIT development is scarce. There is some evidence that intestinal development may be benefited. Leucine supplementation increased the activity of trypsin in the small intestine (Cao *et al*. 2019). The supplementation of arginine, alongside increased milk allowance, resulted in greater villus height, villus width, villus height to crypt depth ratio and goblet cell numbers in the duodenum compared to calves receiving glutamine supplementation and no supplementation (van Keulen *et al*. 2020). In the jejunum villus height, villus surface area and villus height to crypt depth ratio was greater in calves supplemented arginine and glutamine, alongside a high milk allowance, compared to calves receiving no supplementation (van Keulen *et al*. 2020). No differences were found with supplementation of glutamine or arginine in the ileum (van Keulen *et al*. 2020). This study investigated the effect of different milk allowances alongside amino acid supplementation, so it is difficult to conclude what extent amino acid supplementation would have on GIT development without differing allowance levels.

In conclusion, the structural development of the rumen is improved via the inclusion of probiotics, prebiotics, synbiotics and butyrate. The ruminal microbiome is improved by the inclusion of probiotics and butyrate. Structural development of the certain areas of the lower GIT is improved by probiotics and prebiotics. Intestinal microbiome is improved by inclusion of probiotics, prebiotics and synbiotics. Amino acids may also be beneficial in GIT development; however, more research is required to conclude this.

2.4 Effect of weaning management factors on gastrointestinal tract development

Milk weaning in cattle is "the gradual reduction in milk intake, accompanied by increasing social independence from the dam and increasing intake of solid feed" as defined by Weary *et al*. (2008). This definition does not accurately describe weaning under artificial conditions, common in New Zealand. Artificial weaning could be considered in two steps: the removal of the calf from the dam (weaning from dam), involving the introduction into new social groups and the transition from liquid to solid feeds (milk weaning). When considering the two steps of the weaning process under artificial rearing conditions, a more suitable definition would be; the adaptation of the calf to new social and physical environments following removal from the dam and the latter transition from liquid to solid feed (often forages) occurring earlier than the calf's natural weaning age. Weaning age and weaning method are two management practices that can influence the weaning success for better welfare and performance of calves during the immediate post-weaning period.

2.4.1 Weaning age

Early milk weaning, under artificial rearing conditions, is generally practiced in dairy systems to save costly labour, and milk or MR. To achieve early milk weaning, it is necessary to reduce the milk allowance to promote solid feed consumption early in life (Khan *et al*. 2016). Earlier empirical evidence suggests calves can start fermenting feed and utilise VFAs for energy at as young as three weeks old (Matin *et al*. 1959), allowing calves to cope with early milk weaning. However, more recent evidence suggests that older weaned calves, at eight weeks of age, were better able to cope compared to calves weaned at six weeks (Eckert *et al*. 2015). Understanding how weaning age can affect the development of the gastrointestinal tract may allow us to better estimate an ideal milk weaning age for artificially reared calves.

There is conflicting evidence in the literature on the effect of weaning age in terms of structural rumen development. For example, Kehoe *et al*. (2007) reported no difference in rumen papillae length and width, and rumen wall thickness when measured at eight weeks between calves weaned at three, four, five or six weeks of age. Similarly, Carballo *et al*. (2019a) found little differences in empty rumen weight, papillae density, surface area ratio, length, and width, as well as muscle layer thickness and epithelial cell thickness when early weaned lambs (four weeks) were compared to control lambs (six weeks). In contrast, other studies have found greater papillae lengths (Zitnan *et al*. 1999; Stobo *et al*. 1966), widths (Zitnan *et al*. 1999) and density (Stobo *et al*. 1966), for earlier weaned calves. This contrasting evidence may be due to the differences in age of slaughter, timing between treatments to implement milk weaning, milk weaning protocols and amount of milk provided to calves.

There is evidence that growth and development of the rumen continues, regardless of milk weaning. For example, rumen weight relative to body weight continued to increase until 17 weeks of age (Schwarzkopf *et al*. 2022). This suggests that early milk weaning may not provide improved conditions for rumen development, as development continues past the age of early weaning anyway, and that delayed milk weaning will not delay rumen development (Abdelsattar *et al*. 2022). This idea was also supported by Silper *et al*. (2014) who found that rumen papillae, non-keratinised epithelium and keratin layer thickness increased with age. Additionally, it was supported by Abdelsattar *et al*. (2022) that papillae height and width, muscle layer thickness and epithelial thickness also had a linear relationship with age.

Volatile fatty acid concentrations (Graham and Simon 2005), BHB concentration (Kargar and Kanani 2019), and pH (Mirzaei *et al*. 2015) can be used as indicators of rumen development. Weaning age was found to have no effect on VFA concentration in the rumen (Schwarzkopf *et al*. 2022), with no difference being found between weaning ages at four weeks and 16 weeks of age (Carballo *et al*. 2019a). Silper *et al*. (2014) showed that proportions of VFAs change as the calf ages and were not different at the end of the study (20 weeks), therefore, these changes may be a result of age, and be independent of milk weaning age. A higher concentration of BHB was found in early weaned (eight weeks), compared to late weaned (13 weeks) calves, which indicates greater ruminal metabolic development (McCoard *et al*. 2019). Similar findings were also reported in lambs (Carballo *et al*. 2019a). Weaning age had no significant effect on ruminal pH at 20 weeks, when calves were weaned at seven weeks compared to 17 weeks (Schwarzkopf *et al*. 2022). The effects of weaning age on both VFA production and its neutralisation and absorption in the rumen are not well studied in developing calves. Further, many dietary and management factors that influence solid feed intake and rumen fermentation are variable across studies. It is difficult to conclude to what extent weaning influences the concentration of VFA and pH of the developing rumen.

Microbial colonisation, or changes to the microbial population, can alter the VFA production and concentrations in the rumen (Lukitawesa *et al*. 2020). Meale *et al*. (2017a) proposed that weaning age influences the microbiome composition in the rumen. Calves weaned at six weeks of age had a greater diversity (Meale *et al*. 2017b) and a differing proportion of microorganisms (Dong *et al*. 2019) compared to calves weaned at eight weeks. This difference may be a result of a more gradual shift in microbial diversity because of a later weaning age (Meale *et al*. 2017a). The differences may also be a result of changes in the microbial population that are naturally occurring with age, with key changes occurring at three time points: 1-30 days old, 30-100 days old and 100-830 days old (Furman *et al*. 2020). This suggests that different ages of weaning may result in milk weaning occurring before/after these key time points. This may lead to the differences in microbial population due to different distributions of microorganisms being present during these time points.

Irrespective of the weaning age, calves eventually reach a 'mature' state (Steele *et al*. 2017). Schwarzkopf *et al*. (2022) found that rumination variables (chewing behaviour, rumen fill, rumen sound), VFA concentration and pH of ruminal fluid showed no significant difference between weaning ages of seven weeks versus 17 weeks, by 20 weeks. This suggests that calves will reach a competent state of rumen development and concluded that a "functional ruminant status" is reached at 11 weeks of age. A similar finding was reported by Abdelsattar *et al*. (2022), with rumen development of goats ongoing until 70 days (10 weeks), and this was in agreeance with Jiao *et al*. (2015) who conclude that both the functional and anatomical development of the rumen is achieved after two months (eight weeks of age). However, calves that were weaned at seven weeks showed signs of impaired rumen function and inadequately developed metabolic adaptations (Schwarzkopf *et al*. 2022). This suggests that weaning at or prior to seven weeks does not provide enough time to reach a mature rumen state, especially when compared to the later weaned calves (17 weeks) which showed no signs of impaired rumen development (Schwarzkopf *et al*. 2022). In summary, calves reach a mature state, likely around 11 weeks of age, this is irrespective of weaning age. However, weaning prior to seven weeks of age may result in impaired rumen function due to a lack of adequate development.

There is contrasting evidence on the effect of weaning age effect on the development of the rumen. In terms of the structural development of the rumen, these differences in results may be due to when the measurement of rumen traits is done. In terms of metabolic development, there appears to be some evidence that earlier weaned calves have improved metabolic development. There were also differences found for ruminal microbial populations in calves weaned at different ages. However, some of these changes in the structure and functional traits of the rumen may be, at least partially, occurring naturally with age, independent of weaning. However, weaning age delay or fast tracking it affects the functional, metabolic, and structural development of the rumen. The effects of weaning age on rumen development are influenced by solid feed consumption.

Research surrounding the effect of weaning age on the development of the lower GIT appears to be significantly less published than that on the rumen. A study conducted with lambs, reported no difference in intestinal morphology at 16 weeks of age in artificially reared lambs weaned at four versus six weeks of age (McCoard *et al*. 2020). Contrarily, Abdelsattar *et al*. (2022) reported a quadratic increase in colonic mucosal thickness, colonic muscle layer thickness and jejunal crypt depth, a linear increase in ileal crypt depth and an increase cubically in ileal epithelial thickness and muscle layer thickness with age, from one day old to 84 days old. However, Abdelsattar *et al*. 2022 provided data on ontogenetic development of intestine where animals were weaned at same age and the study was conducted on goats, so results may not be the same in calves. There is also evidence that permeability may be influenced by age. Permeability of the GIT reduces as age

increases (Wood *et al*. 2015). This may be a result of dietary changes at weaning as they lead to a shift from energy being acquired via intestinal absorption to acquirement via ruminal microbial fermentation and the subsequent absorption of the end products (Eckert *et al*. 2015; Meale *et al*. 2017a).

Microbial population in the lower GIT may be influenced by both age and weaning age as a significant interaction for time and weaning age was reported for the pH of faeces (Schwarzkopf *et al*. 2022). Changes in faecal pH have been explained by changes in the lower GIT microbiome (Osuka *et al*. 2012). It was also reported that later weaned calves (17 weeks) microorganisms had greater adaptability to the changes in diet due to weaning (Amin *et al*. 2023). Amin *et al*. (2023) also suggested that maturation of the GIT is dependent on age. Differences in intestinal development may be seen across different weaning ages due to variability in GIT maturation. However, Meale *et al*. (2017a) found that there was no change in the relative abundance of intestinal phyla between weaning ages six weeks or eight weeks, due to a lack of change in alpha diversity of faecal microbiota.

In summary, empirical research about the effects of weaning age on the structural development of the ruminant lower GIT is scarce. There has been no difference reported in sheep but there does appear to be a relationship with age with goats. Permeability of the lower GIT may decrease with age, but this does not seem to be a direct result of weaning age. There is contrasting evidence for the effect of weaning age on the lower GIT microbiome. Later weaned calves have greater adaptability of their microbiome than earlier weaned calves, however, the abundance of types of microorganisms is not different between weaning ages. Some of the differences in lower GIT microbiome may be due to an interaction effect of time and weaning age and not a direct result of weaning age.

In conclusion, the rumen appears to be influenced by weaning age more than the lower GIT is. Differences in the rumen, as a result of weaning age, may include metabolic development, microbial population, as well as some structural traits. However, there are some disagreements as to whether structural traits are truly influenced by weaning age. It does appear that by approximately 11 weeks of age, all animals will reach what can be considered a mature state and any differences due to weaning age will not be present. The influence of weaning age on lower GIT development is unclear. Structural development may be influenced more by age, independent of weaning age. The microbiome of the lower GIT has increased adaptability in later weaned calves, but there is no change in relative abundances of phyla.

2.4.2 Weaning method

The conventional weaning method typically follows a regime of restricted milk allowance to around 10% of calf birthweight throughout the whole milk feeding period (Khan *et al*. 2007a, 2007b; Silper *et al*. 2014; Steele

et al. 2017). Under conventional and restricted milk feeding systems, to encourage the calf to consume solid feed, milk weaning is generally abrupt, as milk is suddenly no longer offered, to encourage the calf to consume solid feed (Khan *et al*. 2011b). Research undertaken in the last two decades has generated evidence that greater or *ad libitum* milk allowance can improve calf welfare and future performance (Jasper and Weary 2002; Khan *et al*. 2011b, Soberon and Van Amburgh 2013). This evidence triggered a change in the farm practice advice to provide increasing amounts of milk to calves. However, provision of greater milk allowance reduces the solid feed intake and therefore delays the rumen development (Khan *et al*. 2011b, 2016). Khan *et al*. (2007, 2008) first proposed the idea of gradual weaning for *ad libitum* milk fed calves namely the step-down weaning method. The step-down weaning method involves a gradual reduction in milk allowance over a specific time period (Mirzaei *et al*. 2020; Meale *et al*. 2017b) to increase solid feed consumption before complete weaning from milk is achieved. Others have tested and refined the gradual weaning / step down weaning method at different ages, duration and magnitude of milk reduction overtime (Sweeney *et al*. 2010; Omidi-Mirzaei *et al*. 2015 Dennis *et al*. 2018; Welboren *et al*. 2019). A stepwise or linear decrease in milk allowance helps trigger solid feed intake, promotes establishment of rumen fermentation, and prepare the developing calves to successfully transition to solid feeds (Khan *et al*. 2016).

Some of the published research suggests that the use of different weaning methods results in no change in rumen development (Roth *et al*. 2009). This was shown by a lack of differences in papillae length between concentrate-dependent weaning and conventional weaning (Roth *et al*. 2009), papillae length, papillae width, empty weight of the rumen (Steele *et al*. 2017) and ruminal microbiome population (Meale *et al*. 2016) between abrupt weaning and gradual (step-down) weaning. However, other evidence suggests that the step-down weaning method improved ruminal development compared to the abrupt weaning method (Silper *et al*. 2014). Weight of the forestomach, rumen wall thickness, papillae length, width, and density in calves on a step-down weaning method was improved compared to a conventional method (Khan *et al*. 2007a). The rumen had improved metabolic (Steele *et al*. 2017; Khan *et al*. 2007a) and physical development (Khan *et al*. 2007a, 2007b) when gradual weaning (step-down) was compared to abrupt weaning (conventional). The use of step-down weaning protocol also results in a more gradual shift in the microbiome towards the mature state (Meale *et al*. 2017b), however, this was in combination with delayed age at weaning, so it is unclear if it is a direct result of the weaning method. BHB was higher in gradually weaned calves, compared to abruptly weaned calves (Steele *et al*. 2017; Khan *et al*. 2007a), which suggests that weaning method may influence rumen development, and fermentation in particular. The positive effect of gradual weaning method on ruminal fermentation was also supported by an increase in fermentation end products, particularly total VFA, acetate and propionate concentrations in the rumen (Steele *et al*. 2017; Khan *et al*. 2007a). BHB levels were also higher for the step-down weaning method compared to the step-up step-down method (Omidi-Mirzaei *et al*. 2015) and when a linear step-down method (percentage decrease) was compared to more individually

based step-down methods (Welboren *et al*. 2019). While there is evidence that weaning method can affect rumen development, there is conflicting evidence whether differences are maintained after weaning (Silper *et al*. 2014; Meale *et al*. 2017b) or not (Steele *et al*. 2017; Khan *et al*. 2007a, 2007b; Omidi-Mirziaei *et al*. 2015).

The literature is lacking on the effects of weaning method on the development of lower digestive tract in calves. There was found to be no difference in the length or weight of the small intestine, caecum, and large intestine (Steele *et al*. 2017). However, there was an increase in faecal starch content in abrupt weaned calves, compared to gradually weaned calves which was proposed to be caused by a lack of gastrointestinal tract adaptation (Steele *et al*. 2017). Weaning method appears to have little effect on the development of the lower GIT.

In conclusion, weaning method appears to influence the rumen more so than the lower GIT. However, it is not clear whether the differences in rumen development are maintained post weaning. Gradual weaning methods, such as the step-down method, may help to wean calves successfully at younger ages. However, there are many other factors that may influence the success of early weaning using a step-down method, particularly the provision of solid feeds such as concentrates.

2.5 Research opportunity

Much of the empirical research surrounding weaning manipulations, such age weaning method and weaning age, were carried out under conditions that are not always applicable to the commercial system in New Zealand. Some studies were completed indoor or provided concentrates prior to/during weaning, as shown in Table 2.7. New Zealand farming systems are mostly extensive farming systems. This means that calves are mostly raised outdoors and their main, if not only, solid feed is pasture, however, concentrate feeds are not uncommon. Therefore, it is difficult to conclude whether results from previous research would have similar influence under commercial calf rearing systems in New Zealand. Research on the effect of weaning age and step-down weaning methods, on calves reared outdoors on pasture and without the provision of solid feeds, will more closely mimic rearing system in New Zealand. Hence the need for the study presented in this thesis. The findings will be beneficial to help farmers make informed decisions about the practices they implement for weaning age and weaning method. It will help to understand whether early weaning, a common weaning method, can be beneficial for the development of the GIT, and if weaning without concentrate feeds is possible without restricting/delaying GIT development.

Table 2.7 Differing factors of weaning age and weaning method studies.

Factor	Studies
Indoor	Kehoe *et al*. (2007); Carballo *et al*. (2019a); Zitnan *et al*. (1999); Schwarzkopf *et al*. (2022); Abdelsattar *et al*. (2022); McCoard *et al*. (2019); Dong *et al*. (2019); Furman *et al*. (2020); Jiao *et al*. (2015); McCoard *et al*. (2020); Wood *et al*. (2015); Amin *et al*. (2023); Roth *et al*. (2009); Steele *et al*. (2017); Khan *et al*. (2007a, (2007b); Omidi-Mirzaei *et al*. (2015); Welboren *et al*. (2019); Schäff *et al*. (2018).
Solid feed provision	Kehoe *et al*. (2007); Carballo *et al*. (2019a); Zitnan *et al*. (1999); Stobo *et al*. (1966); Schwarzkopf *et al*. (2022); Abdelsattar *et al*. (2022); Silper *et al*. (2014); Meale *et al*. (2017b); Dong *et al*. (2019); Furman *et al*. (2020); Jiao *et al*. (2015); McCoard *et al*. (2020); Wood *et al*. (2015); Amin *et al*. (2023); Roth *et al*. (2009); Steele *et al*. (2017); Meale *et al*. (2016); Khan *et al*. (2007a, 2007b); Omidi-Mirzaei *et al*. (2015); Welboren *et al*. (2019); Schäff *et al*. (2018)

Chapter 3: Material and Methods

3.1 Animals

Friesian-Hereford cross calves (n=72 male) were sourced from two commercial dairy farms; Poplar farm (Foxton, Manawatu, New Zealand), and Sanderry farm (Santoff, Manawatu, New Zealand). On each farm, calves were collected twice daily from calving areas (paddocks), ear tagged, and their naval were sprayed with disinfectant. All calves received two doses of 2 L of colostrum within the first 24 hours of their birth. Calves were housed in group pens bedded with woodchips at their farm of origin, supplied free access to drinking water and offered transition milk (3 L) twice daily during the first three weeks (±2 days) of life as per normal farm practice on New Zealand dairy farms. Calves were offered *ad libitum* access to grass hay in their respective pens. No other solid feed was offered to calves during the first three weeks of life. At the end of 3 weeks of age, calves were weighed and checked for any signs of abnormality (diarrhoea, nasal discharge, umbilical inflammation). Only healthy calves were sourced and transported (10-15 km) to AgResearch Aorangi Farm (experimental facility), Manawatu, New Zealand for the study. All procedures used in the animal study were approved (15591) by the Ruakura Animal ethics committee, AgResearch Limited, New Zealand.

Calves were transported to experimental facility in three batches. The calves entered the trial at a mean age of 21.3 ± 2.9 days old. Calves were divided into two treatment groups, early weaned (weaned at 10 weeks of age) and late weaned (weaned at 20 weeks of age). Each treatment group (36 calves per treatment) was further split into six sub-groups (large paddocks; 1.7 ± 0.2 hectare/paddock, with ryegrass clover mixed pasture), each consisting of 6 calves. Within each arrival batch, treatments were balanced by farm of origin and sex of the calf. Calves remained in their respective paddocks until humanely slaughtered.

From the start of week four (± 2 days), all calves were offered a MR (Sprayfo Rosso, Trouw Nutrition, Deventer, The Netherlands). All calves were transitioned gradually to a full dose of MR (1000 g/calf/day). On day one of the study, all calves received 600 g MR, across two feeds (morning and afternoon), and the daily amount of MR offered was increased by 100 g/day to achieve a daily feeding rate of 1000 g on day five of the study. Thereafter, both early, and late weaned calves were offered 1000g/calf/day MR until the end of weeks seven and 17 of age, respectively. MR allowance was reduced gradually over three weeks (33% per week) starting from weeks eight and 18 and milk weaning was completed by the end of weeks 10 and 20 of calf age for early and late weaned calves, respectively. As a prophylactic to coccidiosis, Coccistop® (Nutritech, Auckland, NZ) was mixed with MR (0.425g per calf per day; at morning feeding) and given to all calves until 10 weeks of their age. Calves were kept in the respective paddocks on a pasture-only diet to allow sampling and data collection until 30 weeks of their age, if not humanely slaughtered prior. MR was mixed in a mobile milk feeder (Stallion MT500) and dispensed into a 13-teat compartmentalised fence hung calfateria (Stallion FC13) at each groups paddock. Milk was delivered to calves at a temperature of 37 ± 2 °C. The mobile feeder

and calfateria's were washed and airdried after each feed. The mobile feeder was also thoroughly washed with Saniwise and/or dishwashing liquid and water blasted once-a-week. Individual animal behaviour at the feeder was observed by the farm staff to assure that all calves were drinking. Calves were monitored daily for signs of lameness, wounds, discharge from the eyes or nose, and diarrhoea. Sick calves were treated in consultation with the farm veterinarian.

3.2 Slaughtering

All calves (n = 72; 36 early and 36 late weaned) were humanely euthanised, at three time points during the trial (12 early and 12 late weaned per time point); when the calves reached 10 (10.0 ± 0.4) weeks, 20 (20.1 ± 0.5) weeks, and 30 (30.1 ± 0.4) weeks of age. Calves were transported (20km) on the morning of slaughter from Aorangi Research Farm to AgResearch Grasslands, via a commercial transport agency. All calves were euthanised by captive bolt stunning and exsanguination by a trained AgResearch Technician. The calves were slaughtered individually to allow quick and smooth collection of tissue samples.

Once euthanised, calves were hung on hooks from their rear limbs. The visceral organs were immediately removed and dissected, and the carcass disposed of after recording empty carcass weight measurements. Carcass weight included the hide, hooves, head, and tail.

3.3 Sampling and processing

Once the digestive system was removed from the animal, the forestomach (reticulorumen, omasum, and abomasum) was tied off and dissected away from the rest of the GIT. The duodenum, jejunum, ileum, caecum, and colon were clamped off, before being individually dissected. The reticulorumen, omasum, and abomasum were also tied off from one another before being individually dissected. Each area of the digestive tract was then emptied and rinsed with cold water, and their weights recorded.

Tissue samples were collected from the rumen (ventral sac (VS) and dorsal sac (DS)), duodenum (approximately 10cm posterior of the pylorus), jejunum (approximately 30cm posterior to the mesenteric junction) (van Keulen *et al*. 2020) and caecum. Two samples of approximately 1cm x 1cm were collected from each area and immediately placed in pottles containing 10% neutral buffered formalin (>10x excess volume of the tissue) and sent to the Massey University Histology for slide creation. Histology slices were prepared by the Histopathology Laboratory of the School of Veterinary Sciences at Massey University (Palmerston North, New Zealand). The segments were dehydrated overnight through graded levels of alcohol (70%, 95% and absolute alcohol) at ambient temperature, cleared in xylene and impregnated with Histosec pastilles

(Merck, Darmstadt, Germany) under pressure at 60°C (Excelsior ES Tissue Processor, ThermoFisher Scientific, MA, USA). The segments were then embedded in wax (HistoStar Embedding, ThermoFisher Scientific, MA, USA), sectioned to three-micrometer slices (Rotary Microtome, microTec, Duisburg, Germany; and RM2235 Rotary Microtome, Leica Microsystems, Wetzlar, Germany), and mounted on a slide. The sections on slides were placed in xylene and ethanol for de-paraffinization and rehydration. The cut sections were stained with a Mayer's-Harris hematoxylin mixture and Eosin stain (Autostainer XL, Leica Microsystems, Wetzlar, Germany). These slides were then use for microscopy.

3.4 Histomorphology

Microscope slides were analysed using an Olympus BH2 light microscope and images captured using Jenoptik ARKTUR - USB3.0 Progres Gryphax CMOS Colour Camera. Measurements were made using graphics tools in the camera software (Gryphax Version 2.2.0. 1234 Windows 64 Bit). In total, 59 slides were analysed from the rumen VS, 71 slides from the rumen DS, 69 slides from the duodenum, 72 slides from the jejunum and, 72 slides from the caecum.

3.4.1 Rumen

Samples from the VS and DS were analysed to measure papillae length and papillae width. Papillae width was measured at the base of the papillae and papillae length was measure from the base of the papillae (where width is measure from) to the top of the papillae, as shown in Figure 3.1 (Carballo 2019b).

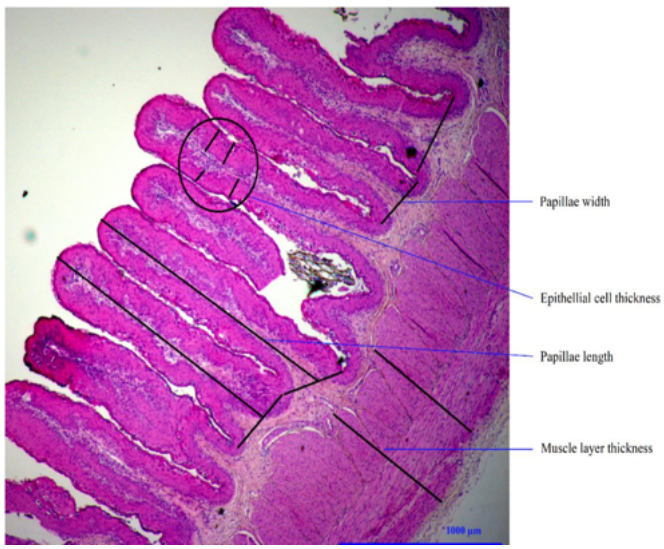

Figure 3.1 Morphological analysis of the rumen. Papillae length was measured at the base of the papillae and papillae length was measure from the base (where width was measured) to the top of papillae.

3.4.2 Small intestine

Tissues samples from the small intestine (duodenum and jejunum) were analysed to measure villus height (VH), villus width (VW), crypt depth (CD) and layer depth. VH was measured from the beginning of the villus-crypt transition to the apex of the villus. CD was measured beginning at the villus-crypt transition to the muscle layer (its base) (van Keulen *et al*. 2020), as show in Figure 3.2 (Adelman *et al*. 2018). Villus width was measured at the base region of the villus as shown in Figure 3.3 (van Keulen *et al*. 2020). Layer depth was measured from the apex of the villus to the muscularis mucosae (base of the crypt). Rinsing of the tissues prior to analysis resulted in damage to some of the villi, layer depth was measured to minimise inconsistencies as a result of damage due to rinsing. However, the rinsing of the tissue was done for consistency in organ weight measurements and show potential treatment differences that may not have been seen if tissues were not rinsed. Villus height to crypt depth ratio (%) was also measured using the following equation: **villus height / crypt depth** (Zhang *et al*. 2017).

Figure 3.2 Morphological analysis of the small intestine. Villus height was measured from the villus-crypt transition to the apex of the villus, and crypt depth from the villus-crypt transition to the muscle layer (Adelman *et al*. 2018)..

Figure 3.3 Morphological analysis of the small intestine. Villus width (VW) was measured at the base region of the villus. Villus height (VH) was measured from the villus-crypt transition to the apex of the villus. Crypt depth (CD) was measured from the villus-crypt transition to the muscle layer (ML). Layer depth was measured from the apex of the villus to the muscle layer (van Keulen *et al*. 2020).

3.4.3 Caecum

Tissue samples from the caecum were analysed to measure crypt depth. Crypt depth was measured from the base of the crypt to the top of the crypt (Hatoko *et al*. 2022), as shown in Figure 3.4 (Adelman *et al*. 2018).

Figure 3.4 Morphological analysis of the caecum. Crypt depth was measured from the base of the crypt to the top of the crypt (Adelman *et al*. 2018).

3.5 Statistical analysis

All statistical analysis were performed using SAS software, version 9.4 (SAS Institute INC., Cary, NC, USA). Descriptive statistics (mean, standard deviation, minimum, maximum and coefficient of variation) were obtained using the MEANS procedure. The data of the dependent variables were examined for normal distribution by inspection of Q-Q plots and histograms, and all of them followed a normal distribution, and therefore the data did not require any transformation. Analysis of variance for dependent variables were performed using the MIXED procedure with a linear model that included the fixed effects of treatment, slaughter week and the interaction between treatment and slaughter week. Farm of origin was included in the model as a random effect and weight at slaughter and organ weight were included as covariates. Least squares means and standard errors for the dependent variables for the different levels of the fixed effects were obtained and used for multiple mean comparisons using the Fisher's least significant test. Significant differences between the means were declared at $P<0.05$. Pearson correlation coefficient was obtained using CORR procedure to investigate the association between weight at slaughter and organ weight.

Chapter 4: Results

4.1 Descriptive Statistics

Mean, standard deviation, minimum, maximum, and coefficient of variation for histomorphological traits are shown in Table 4.1. The coefficient of variation for ventral sac papillae length (24%), caecum crypt depth (17%) as well as villus width (23% and 21%) and layer depth (21% and 20%) for the duodenum and jejunum, respectively, are relatively low in comparison to most other traits. Duodenum crypt depth exhibits a very large coefficient of variation (104%).

Table 4.1 Descriptive statistic to summarise measurements for the histomorphological traits of the rumen, duodenum, jejunum and caecum of calves in New Zealand.

Trait[1 2]	N[3]	Mean	SD[3]	Min[3]	Max[3]	CV[3](%)
VS papillae length	59	395.08	179.40	58.00	1137.80	45
VS papillae width	59	31.65	7.55	19.00	66.33	24
DS papillae length	71	214.22	115.27	37.00	521.33	54
DS papillae width	71	25.79	12.03	12.00	102.33	47
Duodenum VH	69	28.28	8.18	16.00	58.00	29
Duodenum VW	69	15.95	3.73	9.00	24.67	23
Duodenum CD	69	41.04	42.63	14.33	231.67	104
Duodenum VH/CD ratio	69	1.41	0.54	0.12	3.05	38
Duodenum LD	69	76.51	15.98	51.33	131.67	21
Jejunum VH	72	32.75	9.34	17.67	56.00	29
Jejunum VH	72	16.97	3.57	10.33	30.33	21
Jejunum CD	72	30.38	9.26	12.33	54.67	30
Jejunum VH/CD ratio	72	1.15	0.41	0.44	2.66	35
Jejunum LD	72	82.02	16.28	58.00	136.67	20
Caecum CD	72	49.56	8.40	31.33	68.00	17

[1]Unit measures for all traits are µm.

[2]VS= ventral sac, DS= dorsal sac, VH= villus height, VW= villus width, CD= crypt depth, LD= layer depth.

[3]N= number of observations, SD= standard deviation, min= minimum, max= maximum.

4.2 Analysis of variance

F-values for histomorphological traits and their associated probabilities for each dependent variable from the analysis of variance are given in Table 4.2. Effect of weaning treatment was significant for caecum crypt

depth, but not for other traits. Effect of slaughter week was significant for ventral sac papillae length, jejunum crypt depth and jejunum layer depth. The effect of interaction between weaning treatment and slaughter week was significant for ventral sac papillae length. Organ weight accounted for the variation in jejunum villus height, jejunum villus height/crypt depth ratio and jejunum layer depth. Slaughter weight did not account for variation for any of the traits.

Table 4.2 F-values for histomorphological traits in the gastrointestinal tract of Hereford-Friesian crossbred male calves that were weaned early and late and slaughtered at 10, 20 and 30 weeks of age in New Zealand.

Trait[1 2]	Weaning age (W)	Slaughtering age (S)	Interaction W × S	Slaughter weight	Organ weight
VS papillae length	2.10	7.86**	3.74*	0.34	0.34
VS papillae width	0.00	0.00	0.93	1.04	1.06
DS papillae length	0.16	0.76	0.80	0.00	0.44
DS papillae width	0.72	1.07	0.06	0.17	0.09
Duodenum VH	0.16	1.59	1.81	0.43	1.93
Duodenum VW	0.42	3.62*	0.24	1.61	1.00
Duodenum CD	2.63	0.56	0.10	2.40	0.01
Duodenum VH/CD ratio	0.21	0.09	1.65	0.00	2.71
Duodenum LD	0.23	2.82	1.59	0.57	0.05
Jejunum VH	1.25	2.24	0.16	0.01	4.41*
Jejunum VW	1.79	2.36	0.53	0.07	0.99
Jejunum CD	1.13	8.55***	0.39	1.27	0.00
Jejunum VH/CD ratio	0.06	1.67	1.33	0.12	4.26*
Jejunum LD	0.03	3.77*	0.34	0.20	5.95*
Caecum CD	4.84*	1.33	0.1	0.3	0.05

[1]Statistical significance is given as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

[2]VS= ventral sac, DS= dorsal sac, VH= villus height, VW= villus width, CD= crypt depth, LD= layer depth.

4.3 Least squares means.

Least squares means and standard errors for each treatment, within slaughter week, for histomorphological traits of the gastrointestinal tract are presented in Table 4.3. Calves that were slaughtered at week 20 in early weaned calves showed higher ventral sac papillae length ($P<0.05$) than late weaned calves. There were no significant differences for traits between levels of other fixed effects. There were no statistically significant differences for any other traits of the gastrointestinal tract.

Table 4.3 Least squares mean (µm) and standard error for histomorphological traits in the ventral sac (VS) and dorsal sac (DS) of the rumen for calves in New Zealand classified into 3 groups for slaughter week (SW) and a further 2 groups for weaning treatment.

	Slaughter age					
	10		20		30	
Trait[1 2]	Early	Late	Early	Late	Early	Late
VS papillae length	164.7±94.3	100.1±103.0	551.4±70.0[a]	340.2±56.6[b]	649.8±125.3	726.7±138.3
VS papillae width	33.3±5.2	30.6±5.7	32.4±4.0	31.2±3.4	29.5±6.8	33.6±7.4
DS papillae length	169.5±49.2	144.5±52.4	200.9±30.3	212.4±26.6	252.0±49.4	291.6±62.8
DS papillae width	20.8±6.9	24.8±7.4	21.6±4.1	23.2±3.6	30.9±7.0	33.4±8.9
Duodenum VH	32.1±4.1	37.4±4.2	30.1±2.6	26.8±2.1	21.6±4.2	22.0±5.3
Duodenum VW	17.8±2.1	16.5±2.1	17.8±1.3	18.0±1.1	13.3±2.1	12.4±2.7
Duodenum CP	16.7±23.3	36.7±24.1	16.7±23.3	44.0±12.0	57.5±24.0	69.1±30.4
Duodenum VH/CD ratio	1.2±0.3	1.5±0.3	1.6±0.2	1.3±0.2	1.3±0.3	1.6±0.4
Duodenum LD	89.1±8.6	92.2±8.8	79.6±5.5	68.2±4.4	64.1±8.8	66.5±11.2
Jejunum VH	38.5±5.1	42.7±5.1	30.1±3.1	32.9±2.6	25.7±5.3	26.7±6.5
Jejunum VW	17.5±1.9	17.5±1.9	15.0±1.2	16.4±1.0	16.6±2.0	18.8±2.5
Jejunum CD	37.0±5.3	37.0±5.3	23.0±3.6	27.3±3.2	26.5±5.4	29.0±6.4
Jejunum VH/CD ratio	1.2±0.2	1.4±0.2	1.4±0.2	1.2±0.1	0.9±0.2	0.9±0.3
Jejunum LD	92.1±8.8	95.5±8.8	78.5±5.3	74.9±4.6	74.3±9.1	76.8±11.2
Caecum CD	55.0±4.7	48.9±4.8	54.3±3.0	50.4±2.6	46.9±4.8	42.6±6.1

[1 a, b] Means with different superscripts within the same row and same slaughter age are significantly different (P<0.05).

[2] VS= ventral sac, DS= dorsal sac, VH= villus height, VW= villus width, CD= crypt depth, LD= layer depth.

4.4 Correlations

The Pearson correlation coefficients of slaughter weight on organ weight are presented in Table 4.4 All organ weights appear to have a significant positive relationship with slaughter weight, as slaughter weight increases, organ weight also increases. Rumen reticulum (0.98) and jejunum (0.91) weight have a much stronger relationship with slaughter weight compared to duodenum (0.49) and caecum (0.51) weight.

Table 4.4 Pearson correlation coefficients (r) and p-values between organ weights and slaughter weights in calves.

	Slaughter weight	
Trait	r	P-value
Rumen reticulum weight	0.98	<0.001
Duodenum weight	0.49	<0.001
Jejunum weight	0.91	<0.001
Caecum weight	0.51	<0.001

Chapter 5: Discussion

In calves, both upper and lower GIT is underdeveloped at birth and go through vast physiological and structural developments during the first few months of their life (Khan *et al*. 2016). Good development of the GIT in essential for efficient digestion and absorption of nutrients and the development of the GIT can influence the lifetime performance of the cow (Byrne *et al*. 2022; Heinrichs and Heinrichs 2011; Burggraaf *et al*. 2020; Diao *et al*. 2019). Many early life feeding, and management practices influence development in both upper and lower GIT. However, weaning age has a substantial effect on the development because milk weaning drastically influences the solid feed intake (Khan *et al*. 2016) and, therefore, changes the substrate reaching lower and upper GIT. Most of the studies conducted to date evaluated the effects of weaning age and methods in calves reared on concentrate starter diets and scientific literature is limited to explaining the effects of weaning age on epithelial development of grass-fed calves. Understanding the effects of weaning age on the development of the epithelium could help improve the weaning and post-weaning performance of young animals in grazing systems. To the best of my knowledge, this is the first study that has evaluated the effects of weaning age on the histomorphology of the upper and lower digestive tract in grass-fed dairy-beef cattle. It was hypothesised that weaning calves from milk replacer at 10 versus 20 weeks of age will result in differences in epithelial development in both the upper and lower GIT. These differences will persist at 30 weeks of age; however, differences will be reduced.

5.1 Rumen epithelial development

In this study, the overall average papillae length for all ages, across all slaughter weeks, was 396 µm (0.396 mm) in the VS and 214 µm (0.214 mm) in the DS. These values were significantly smaller than the average papillae length in the rumen VS reported for mature ruminants (10-15 mm; Graham and Simmons 2005) and in 5-week-old calves (0.94-1.25 mm; Zitnan *et al*. 2005). My results were the average of papillae lengths from 10, 20 and 30-week-old calves which may account for some differences. However, even the maximum VS papillae length of 1138 µm (1.138 mm) was significantly smaller than some previously recorded averages. In the current study, the calves were fed a high level of milk replacer (1kg MR powder) and reared without any concentrate starter diet, which is different from the studies reported in the literature. High milk replacer feeding can delay the initiation of solid feed intake (Sweeney *et al*. 2010) and therefore compromise the epithelial development (Khan *et al*. 2011a). Pasture feeding instead of concentrate can further reduce the substrate availability to trigger the development of rumen epithelium to papillae (Khan *et al*. 2016), due to reduced butyrate in the rumen (Poier *et al*. 2022; Heinrichs and Lesmeister 2004; Mentschel *et al*. 2001). In the current study and Zitnan *et al*. (2005) the specific location of tissue samples, other than VS and DS, were not specified. Whereas Graham and Simmons. (2005) collected VS tissue samples ~10cm from the left longitudinal groove. The differences in papillae length observed in the current and previous studies may be explained by methodological differences between studies, including the site of tissue sampling from the

rumen and measuring techniques. Lastly, the dairy-beef genetics used in the New Zealand farming system, and in this study, is different from European dairy and beef breeds, used in the other reported studies (Graham and Simmons 2005; Zitnan *et al*. 2005). In this study, Friesian-Hereford cross calves were used, whereas Zitnan *et al*. (2005) used German Holstein calves and Graham and Simmon (2005) collected rumens from commercial slaughterhouses in the United Kingdom and breed was not distinguished. Differences in cow breed may have influenced the epithelial development and therefore average papillae lengths.

The effect of slaughter age was significant for VS papillae length ($P<0.001$). Papillae length was lowest at 10 weeks and increased with successive slaughter weeks. The interaction between weaning age and slaughter age was also significant for VS papillae length ($P<0.05$). At slaughter week 10 early weaned calves had numerically higher papillae lengths than late weaned calves and significantly higher at 20 weeks. However, at 30 weeks, late weaned calves had numerically higher papillae lengths than early weaned calves. The effect of age on papillae lengths has been portrayed in the literature, with both Silper *et al*. (2014) and Abdelsattar *et al*. (2022) observing increased rumen papillae length with increasing age. However, weaning age may also account for some of these differences. This has been reported in the literature with greater papillae length in early weaned calves (Zitnan al. 1999; Stobo *et al*. 1966) and more recently in lambs (Carballo *et al*. 2019a). These agreeing studies provided concentrates and the study was completed indoors. Our results suggest that rumen papillae length was not hindered due to lack of concentrate provision. However, it is important to consider that both weaning ages in these studies was significantly younger than in the current study. As discussed in previous sections, solid feed provision can initiate differentiation of the rumen epithelium into papillae (Kertz and Loften 2013; Baldwin *et al*. 2004). The differences seen at 20 weeks was likely a result of vast differences in diet. Early weaning calves would have been exclusively consuming forage for 10 weeks, whereas late weaned calves had only just finished step-down weaning from milk and would have been consuming significantly less forage. Consumption of forage results in rumen fermentation (Baldwin *et al*. 2004) and the VFAs produced from fermentation, alongside feed bulk trigger rumen development (Khan *et al*. 2016), so it is expected that characteristics of the rumen are more developed in calves consuming higher amounts of solid feed, early weaned. Interestingly, papillae length in the DS was not affected by any of the independent variables (weaning age, slaughter age, interaction of weaning age x slaughter age, slaughter weight, organ weight) and it was shown that mean papillae length was lower in this location when compared to the VS. Carballo *et al*. (2019a) had similar results in lambs with VS papillae length being numerically greater than DS between treatments and across most time points. Other literature tended to discuss papillae development, generally, rather than exploring the development in different areas. The difference in papillae height between the two areas of the rumen may be due to slightly varying functions. The functions of papillae are to increase the surface area allowing increased absorption of fermentation end products (Heinrichs 2005). Therefore, it could be suggested that more absorption occurs in the ventral sac of the rumen because of the

increased papillae length. At 30 weeks both DS and VS papillae lengths are greater in late weaned calves. This suggests that calves in the current study may reach the 'mature' rumen state as suggested by Steele *et al*. (2017). However, other literature has suggested that this state is reached before 30 weeks of age (Abdelsattar *et al*. 2022; Jiao *et al*. 2015). This difference may be a result of differing milk feeding periods and lack of concentrate allowance.

5.2 Lower gastronintestinal epithelial development

The effect of slaughter was significant for duodenum VW ($P<0.05$). For early weaned calves, VW remains the same at 10 and 20 weeks but was reduced at 30 weeks. There was a slight increase at 20 weeks, for late weaned calves, but also reduces at 30 weeks. The factors that can influence VW and the importance of VW are very scarce. However, it is involved in the surface area (SA) calculations for the villi (Jing *et al*. 2022):

SA= Villus height x villus width x 2

Greater VW increases the SA of the villus, allowing for greater absorption of nutrients (Yang and Nam 2022; Streckfus 2022). Villus SA is responsible for capacity of glucose absorption which is an important energy source (Jing *et al*. 2022). Low energy intakes resulted in increased SA of the villi throughout the intestines in sheep (Jing *et al*. 2022). This increase in SA may be an adaptation to absorb more energy from glucose when required. Therefore, it could be proposed that increases in VW in the duodenum are a result of changes or in energy level of the diet or adaptation to changes it dietary source. However, Schäff *et al*. (2018) study results do not agree with this theory as milk allowance had a positive relationship with jejunum villus SA of five-week-old calves and found no difference in duodenum villus SA. It is difficult to conclude if either of the results from these studies are comparable to the current study as Jing *et al*. (2022) study was carried out on mature sheep (1.5 years old) so therefore milk consumption was not a factor and Schäff *et al*. (2018) provided *ad libitum* concentrate feed to the calves.

The effect of slaughter age was highly significant for jejunum CD ($P<0.001$). Adbelsattar *et al*. (2022) provided evidence that crypt depth changes with age but suggested the increase occurs in a quadratic pattern. Quadratic growth is defined as a "constant rate of rate of change" ("*Quadratic Growth"* n.d.). The jejunum CD results from the current study do not follow a quadratic pattern. Jejunum CD decreases from 10 weeks to 20 weeks and slightly increases at 30 weeks, across both weaning ages. Schäff *et al*. (2018) found that calves on *ad libitum* milk diets had reduced CD compared to a restricted diet, however, this was not a result of feed intensity ($P >0.05$) and therefore likely not a result of solid feed intake either. Therefore, other factors associated with different ages may be causing this difference. Some literature suggests that CD is influenced by particles size in chickens (Ariyadi *et al*. 2019; Tejeda and Kim 2021). The particle size difference between MR (10-250 µm; Pugliese *et al*. 2017) and pasture (200-1200 µm; Martz and Belyea 1986) may be a cause for

jejunum CD difference as the proportion of these changes as the animal ages. The ruminant lower gut is relatively analogous to that of a monogastric (e.g., Chicken) (Gäbel *et al*. 2002; Steele *et al*. 2016; Kaba *et al*. 2018) so we may expect similar results, but more research would be required to confidently conclude that crypt depth differences across different ages was a result of differences in particle size.

Morphological traits of the jejunum were also influenced by jejunum weight: layer depth ($P<0.05$), VH ($P<0.05$), and VH/CD ratio ($P <0.05$). This was not unexpected as increases in layer depth and height are a result of greater tissue formation, which would contribute to the overall weight of the organ. However, it was unexpected that we did not see the same relationship with organ weight for other areas of the GIT.

The effect of slaughter age was also significant for jejunum layer depth ($P<0.05$). There was a decrease in layer depth from 10 to 20 weeks and a small decrease at 30 weeks for early weaned calves and a small increase at 30 weeks for late weaned calves. This pattern of change across the slaughter weeks was similar to that of jejunum CD and VH, excluding early weaned calves at 30 weeks. Layer depth is essentially a combination measurement of VH and CD and due to the significant relationship of jejunum CD with slaughter age and VH with organ weight, it is likely that changes in layer depth were related to CD and VH.

The effect of weaning age was significant for caecum CD ($P<0.05$). Crypt depth decreased across the slaughter weeks for both early weaned calves but increased from 10 to 20 weeks, followed by a decrease at 30 weeks for late weaned calve. The function of the caecum in bovine species appears to be unknown. However, in horses it functions to absorb large amounts of water and electrolytes and carry out microbial digestion before digesta is passed into the colon (Murray and Epstein 2022). While the caecum in horses is significantly larger than in other species (Murray and Epstein 2022), it can be assumed that the function in calves would be similar. The difference in crypt depth between weaning age treatments may be a result of differing diets. Diets differed the most at slaughter week 20, with early weaned calves consuming a pasture only diet for 10 weeks and late weaned calves only just having a high milk allowance removed. A diet containing increased pasture may require increased microbial breakdown, such as in the caecum, resulting in increased availability of water, electrolytes, and nutrients for absorption. The caecum can be considered a part of the large intestine, which has a significant increase in the numbers of goblet cells, relative to the small intestine (Streckfus 2022; Barker *et al*. 2008). Goblet cells are found in the crypts and function to absorb water and nutrients (Streckfus 2022). An increase in crypt depth in the caecum of early weaned calves at 10 weeks and late weaned calves at 20 weeks may be expected to accommodate for an increased requirement of goblet cells as a result of increased pasture intake. However, based on this it may be expected that this difference would not be seen, or differences would be reduced, at 30 weeks. However, the numerical difference between treatments was smallest at 20 weeks.

5.3 Correlation of organ weights with slaughter weight

Rumen reticulum weight was highly correlated (0.98) with slaughter weight, this was expected as the size of the rumen increase as the animal ages (Niehaus 2009; Meale *et al*. 2017b; Warner *et al*. 1956; PennState Extension 2022), and as the animal gets older relative size (weight) of the animal would increase too. This would explain the correlation between all organ weights and slaughter weights. Jejunum is also highly correlated with slaughter weight (0.91), especially when compared to the duodenum (0.49) and caecum (0.51). The lower correlation of the caecum with slaughter weight may be due to the influence of diet. The amount of forage in the diet has been found to be proportional with the size of the caecum (Sreekanth *et al*. 2014). At the different slaughtering weeks there was varying amounts of forage in the diet between treatments, except at 30 weeks, and consequently, may account for the weaker correlation between caecum weight and slaughter weight. The lower correlation of the duodenum may be a result of its function. The duodenum is the major intestinal region for continuing chemical digestion (Beaumont *et al*. 2021; Fish and Burns 2022) and this function is likely more important in the pre-ruminant animal due to a lack of rumen function. Therefore, it could be expected that the duodenum would grow quickly when the calf is young and undergo less changes as the weight of the calf increased. The high correlation between jejunum weight and slaughter weight is not well described in the literature.

In summary, most morphological differences were explained by slaughter age rather than weaning age. However, the effect of weaning age was significant for caecum CD and the interaction of weaning age and slaughter age was significant for VS papillae length. Published literature on the effect of age on the development of the gut is limited and even more so for weaning age. Nonetheless, there are other factors influencing GIT development that are better described in the literature and when relating these to weaning we can understand how weaning age may directly or indirectly influence GIT development. These factors include concentrate intake (or lack thereof in this study), milk allowance, pasture intake, cow breed, and feed particle size. The current study differs to most literature due to the lack of concentrate provision. However, this helps us to understand the implications of calf rearing, in terms of GIT development, under conditions similar to commercial calf rearing conditions in New Zealand.

Chapter 6: Conclusions

The findings of this study indicate that most morphological differences in the GIT are accounted for by slaughter age, rather than weaning age. However, the reason for these differences can often be attributed to the diet being consumed by the different treatment (weaning age) groups at the different slaughter times. This change in diet is in response to weaning and it is not possible to separate weaning age from dietary shift. At week 10, early weaned calves were weaned off of milk and their pasture intake was increasing, while the diet of late weaned calves diet was predominately MR (but have access to pasture). At week 20, early weaned calves had been consuming an entirely pasture based diet for 10 weeks, while late weaned calves had only just been weaned from milk. At week 30, both early and late weaned calves have consumed an entirely pasture based diet for 20 and 10 weeks, respectively. Milk replacer bypasses the rumen and therefore limits the substrate available to initiate rumen development. Whereas, pasture intake increases the amount of substrate entering the rumen, thereby allowing rumen development. Weaning age had a significant effect on caecum crypt depth, as well as VS papillae length through its interaction with slaughter age. Early weaned calves had increased VS length at 20 weeks and caecal crypt depth across all slaughter weeks for both of these traits. The increased papillae length of early weaned at 20 weeks was a result of increased solid feed intake providing increased substrate for rumen development, compared to the limited solid feed intake of late weaned calves. Factors effecting crypt depth are not well explained in the literature. The caecum is involved in further microbial breakdown of feeds, after the rumen, and the differences may also be explained by the differences in dietary shift in response to weaning. The increase in caecum crypt depth for early weaned calves at 10 weeks and increase in late weaned calves at 20 weeks may be to accommodate a requirement for increased goblet cell numbers, which reside in the crypts and are involved in nutrient and water absorption. However, the differences in both morphological traits (VS papillae length and caecum crypt depth) did not persist post-weaning (at slaughter week 30). It is also important to consider that the lack of differences between morphological traits, as a result of weaning age, provides evidence that delaying weaning age does not appear to hinder GIT development. The findings from this study will allow calf rearers in New Zealand to understand the potential implications of manipulating weaning age.

I hypothesised that the greatest difference, between early and late weaned calves will be seen at slaughter week 20, due to the consumption of different diets at this time and that differences will be sustained at 30 weeks, but the differences will not be as large. I accept the first part of the hypothesis as any differences that were seen between treatment groups were seen at slaughter week 20. However, I reject the final part of the hypothesis as differences were not sustained at 30 weeks.

This study successfully reared and weaned calves on a pasture only diet, for both early (10 weeks) and late (20 weeks) calves. This may permit the rearing and weaning of calves without the provision of concentrate feeds, but further investigation on growth performance would be required to conclude this.

Future research is required to extend the knowledge of the effect of weaning age on GIT development, particularly under conditions that are comparable to commercial conditions in New Zealand. Future studies are required to investigate the effects on GIT development when calves are reared and weaned, at the same age, on pasture only diet versus pasture and concentrate diets. As well as studies on the rearing and weaning of grass-fed animals with different genetics on concentrate versus pasture diets. This will help confirm if results are applicable over both dairy and beef systems and potentially overseas, where different genetics may be used. Future studies may also investigate the effect of pasture only weaning and weaning age on long term performance of cows, such as: growth, finishing age, and carcass quality.

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