Effects of bovine, caprine or ovine milk on ileal and colonic microbiota composition and functional potential in an early postnatal model (the pig) of the human infant

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Abstract

The gastrointestinal microbiota plays a vital role during the first 1,000 days of postnatal life. Nutrition is among the foremost determinants of its establishment during this life stage. Breast milk meets all the nutritional needs of infants; however, breastfeeding is not always possible. Infant formulae are typically based on the properties of bovine milk. However, caregivers increasingly seek other ruminant milk to avoid perceived health issues reported with bovine milk. However, the effects of such formulae on the small and large intestinal microbiome in early postnatal life have received limited attention.

The research undertaken aimed to understand the effects of consuming whole fresh bovine, caprine or ovine milk over two weeks on the ileal and colonic microbiota in pigs as a model of the human infant. DNA was extracted from ileal and colonic digesta samples at the end of the intervention. Following quality control checks performed in-house and externally, shotgun metagenomics of the extracted DNA was performed to determine the composition and functional potential of the microbiota in these samples.

Consuming bovine, caprine, or ovine milk did not affect the composition of ileal and colonic microbiota at the phylum and family levels. The relative abundance of some taxa in the ileal and colonic microbiota at the genus level was different between milk groups. However, the functional potential inferred from gene abundances of ileal and colonic microbiota were similar between milk groups. Limitations included a lower than expected ileal sample size and microbiota assessment at a single timepoint.

More research is required to better understand how formulae made up of bovine, caprine, or ovine milk affect the ileal and colonic microbiota and potential benefits for the function of the host tissue locally and elsewhere in the body in pigs as a model of human infants. Equally important, studies investigating the effects of these formulae on the intestinal microbiota are required in human infants.

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Abbreviations

Full terms	Abbreviated terms
Analysis of composition of microbiomes with bias correction	ANCOMBC
Analysis of variance	ANOVA
Body weight	BW
Caesarean section	C-section
Central nervous system	CNS
Gastrointestinal tract	GIT
Germ-free	GF
Human milk oligosaccharide(s)	HMO(s)
Immunoglobulin	Ig
Kyoto Encyclopedia of Genes and Genomes	KEGG
Operational taxonomic unit	OTU
Postnatal days	PND
Ribosomal RNA	rRNA
Receiver operating characteristic	ROC
Sparse partial least squares for discriminant analysis	sPLS-DA

1. Literature Review[1]

1.1. Introduction

The gastrointestinal tract (GIT) represents one of the largest sites of interaction between environmental factors, the host and the microbiota.[1] The human GIT is approximately nine metres long and comprises organs, including the mouth, pharynx, oesophagus, stomach, small intestine and large intestine (Figure 1). Its primary functions are the digestion of foods for nutrient uptake, absorption and metabolism by enterocytes and the clearing of undigested foods and waste products.[2] Appropriate function and coordination of digestion, absorption, motility, secretion, regulation and transport are crucial for keeping host health.[3] These processes require digestive enzymes and transporters, a functional smooth muscle and an intact enteric nervous system.[4] These various processes are heavily influenced by resident microbiota, a collection of microorganisms that is sometimes referred to as an ancillary organ.[5]

1.2. The gastrointestinal microbiota

Within the GIT resides a vast population of bacteria, archaea and eukarya termed the microbiota. They have co-evolved with the host over millions of years to form an intricate and mutually beneficial relationship.[6] Benefits include immune development through strengthening and maintaining epithelial integrity of small and large intestines, with protective effects against pathogens through physical exclusion. The GIT microbiota assists host digestion and utilising nutrients that may otherwise be non-digestible by the host and may also directly or indirectly influence host metabolism.[5,6]

[1] Parts of this chapter has been submitted and is under peer-review by a journal (Appendix A): Mullaney JA, Halliday C, Roy NC, Young W, Altermann E, Kruger MC, Dilger RN & McNabb WC. (2022). Effects of early postnatal life nutritional interventions on host immune- microbiome interactions in the gastrointestinal tract and implications for brain development and function. *Frontiers in Microbiology* (under review).

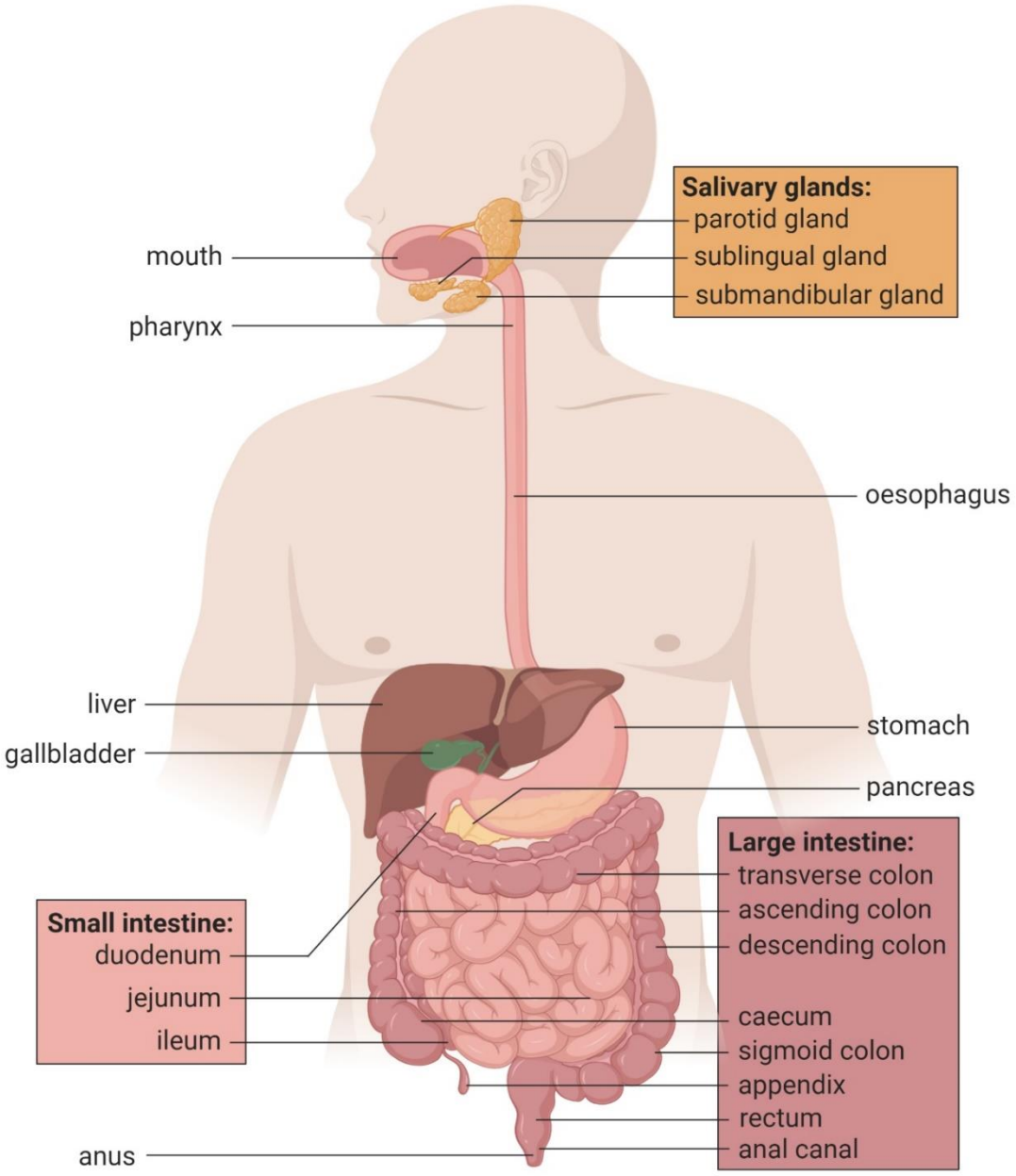

Figure 1. The gastrointestinal system and its organs. Figure adapted from OpenStax College Microbiology (2016)[7] and created with BioRender.com.

Due to the importance of the GIT microbiota, disruption of the colonisation process or composition (dysbiosis) can negatively affect the host.[8] Colonisation of the GIT by a diverse array of microbes is vital during early postnatal life, with the microbiota implicated in having significant effects on host development, for example, the immune system, the cognitive function of the brain and behaviour.[9-13]

Our understanding of how the GIT microbiota interacts with the host has been advanced through research with animal models. Non-human primates are most comparable to humans; they share physiological, metabolic, and genetic similarities.[14,15] However, their cost, lifespan and ethical concerns limit their use for research.[16,17] Other species, such as conventional, germ-free (GF) and gnotobiotic (mice with known microorganisms) rodents, have primarily been used. These are, however, artificial systems where the environmental parameters are controlled.[18,19]

Pigs have shown that they are a more suitable choice over rodents for research focusing on early postnatal life development. Pigs and human infants share similar GIT and brain developmental stages compared to rodents.[15] Pigs also share higher similarities with humans in terms of anatomy and function of the immune system (e.g. pigs possess tonsils, whereas rodents do not).[14] Cannulation is also possible for repeated, stress-free sample collection over the lifetime of the pig.[20,21] Pigs are also the animal model of choice in terms of digestive and associated metabolic processes and nutritional requirements compared with other non-primate animal models.[22] Therefore, using pigs in early postnatal developmental research allows for determining critical developmental time windows that may be sensitive to nutrition intervention.

1.2.1. Colonisation

As reviewed by Walker *et al.*, there is evidence that the infant may not be born with a sterile GIT[23] and may be in contact with the maternal GIT microbiota during the gestation period.[24] This effect has been demonstrated by detecting microbes in the foetal meconium, cord blood and amniotic fluid.[24-26] It is hypothesised that microbiota from the mother may pass into the foetus via the mother's bloodstream.[27] However, evidence supporting the '*in utero* colonisation hypothesis' remains controversial. Authors have argued against the use of molecular methods with insufficient detection power for low-biomass microbial populations and inappropriate controls for contamination with no evidence of bacterial viability.[28] Furthermore, the ability to generate GF rodents via caesarean section

(C-section) remains the strongest evidence against the possibility of microorganisms being present in the foetal environment.

Regardless of the *in-utero* exposure, there is increasing evidence that external factors affecting the mother during pregnancy can influence the development and behaviour of the infant.[29,30] It has been noted that the maternal GIT and vaginal microbiota change during pregnancy, as reviewed by Mueller *et al.*[30] It is unclear what implications these may have on maternal or infant health. It is speculated that these changes may be part of a protective response in preparation for birth by providing the newborn with an 'optimum' inoculum before exposure to external microorganisms.[29,30]

Following birth, there are broadly four phases of microbial colonisation of the GIT, which are affected by different factors: mode of delivery, infant feeding methods, gestational age, weaning period, infection, and antibiotic exposure.[31] This point is further discussed in Section 1.2.4. The first phase of the microbial colonisation of the GIT takes place when the infant is born. Optimally, the newborn is a full-term vaginally delivered baby, which ingests some of the maternal vaginal and faecal bacteria, including bacteria from the *Lactobacillus* and *Bifidobacterium* genera,[32] as it passes through the birth canal.[23] After birth, the infant GIT gradually shifts from an aerobic to an anaerobic environment over days.[33] Facultative anaerobic bacteria, such as those from *Escherichia* and *Enterococcus* genera, are the first bacteria to colonise the infant GIT. As oxygen levels are depleted, obligate anaerobes can also proliferate, some of which include *Bacteroidetes* and *Actinobacteria* phyla.[33-36] At birth until four months of age, the microbiota is continuously stimulated as a result of oral feeding, for example, breast milk or formulae (phase two).

With the introduction of solid foods at weaning, the microbiota receives additional nutrient sources (phase three), allowing for the proliferation and expansion of both density and diversity. By about three years of age, the GIT is colonised by diverse populations of microorganisms (phase four), typically dominated by three bacterial phyla: Firmicutes, Bacteroidetes and Actinobacteria.[37,38] By this stage, the GIT microbial signature stabilises, unless affected by external factors as described in Section

1.2.4.[23,39] In general, the 'normal' GIT microbiota is dominated by anaerobic bacteria, which outnumbers aerobic and facultative anaerobic bacteria by 100- to 1,000-fold. The microbial distribution and diversity increase along the GIT, from the proximal to the distal portions.[40,41]

1.2.2. Composition

Microbial colonisation of the GIT is important for host homeostasis and health.[6,42,43] Consequently, great effort has been put into characterising its composition to determine which microbes are 'beneficial' or 'harmful'. Following the establishment of the individual's microbiota, the composition may change due to internal (e.g., host genetics) or external (e.g., diet or infection) factors.[23,39] With this understanding, it may also be possible to modulate and influence the microbial composition through external factors (primarily diet), particularly in early postnatal life, to optimise host development, maintain homeostasis and reduce the risk of disease.

Originally, the characterisation of the microbial composition of the GIT stemmed from labour-intensive, culture-based methods.[1] These methods enabled the identification of microbes but gave no information regarding their functional capacity. Nowadays, composition is surveyed using culture-independent approaches such as high-throughput sequencing methods.[44,45] The most common approach is targeting the bacterial 16S ribosomal RNA (rRNA) gene, as it is highly conserved across bacteria and archaea.[1] The 16S rRNA gene contains nine hyper variable regions (V1–V9), the sequencing of which allows for the discrimination of many genera.[1] These regions are also flanked by highly conserved regions of DNA, thus allowing for the design of polymerase chain reaction (PCR) primers which bind to these regions.[46] This amplicon sequencing highlights the limitations of culturing methods; for example, the majority (76%) of rRNA sequences obtained from a human faecal sample belonged to novel and uncharacterised species, which so far have not been culturable.[47] A more recent study conducted by Zou *et al.* generated 1,520 new reference genomes cultivated from isolates retrieved from faecal samples in healthy humans, and 264 of these genomes had not been published.[48] Whole-genome shotgun metagenomic methods may also provide a relatively reliable estimate of

microbiota composition and diversity due to the ability to sequence and map to microbial genes and the 16s variable.[44] In contrast, shotgun sequencing reads all the genomic DNA in the sample, as opposed to 16s rRNA sequencing, which is restricted to a specific region of DNA.[49,50] Furthermore, shotgun metagenomics may also distinguish microbial genes involved in metabolic processes to understand their potential functionality and provide insights into a number of processes, including antimicrobial resistance.[46]

However, interpreting this massive amount of data requires substantially more processing power, and incomplete reference databases combined with poor functional characterisation of some genes may limit these metagenomic approaches employed to study the GIT microbiota.[46,51] A combination of 'modern' and traditional microbiology methods (i.e. culture-based methods) have been employed to counter this, in particular to examine the bacteria's virulence and antibiotic susceptibility.[52]

Previous efforts in characterising the GIT microbiota observed country-specific microbial signatures, suggesting that the microbiota composition is heavily influenced by environmental factors, particularly during early life.[49,50,53,54] As a result, the notion of a 'core microbiota,' defined as a set of the same organisms being present in all individuals, has often been disputed.[55] Along the same lines, some researchers theorise that all individuals fall into one of three microbiome clusters, or enterotypes, based on their long-term diet.[56] These enterotypes are also not nation- or continent-specific and are characterised by the abundance of three dominant microbial genera: 1, *Prevotella*; 2, *Bacteroides*; and 3, *Ruminococcus*.[56,57] These enterotypes are associated with differing diets: 1, primarily consuming high-fibre whole-food diets with some refined carbohydrates; 2, high intake of animal proteins and fats, and refined sugars; and 3, a diet rich in dietary fibre and resistant starches. The notion of enterotypes has been challenged, however, due to the temporal fluidity of the microbiota.[58] Furthermore, it has recently been suggested that whilst taxonomic similarities could not be fully distinguished, there are clear functional similarities (functional redundancy) between individuals when comparing protein or metabolite profiles following inferrance from microbial gene

abundances.[59,60] Understanding the 'core functional' bacteria in early postnatal life may provide insight into which functions are conserved.

The composition of the microbiota depends on its biogeographical location within the GIT, where the physiological, chemical, nutritional and immunological properties vary across each region (Figure 2).[1,61,62] Microbial numbers and diversity typically increase from the stomach to the faeces due to the low pH levels found in the stomach and duodenum.[41] High levels of oxygen, enzymes, bile, acids and antimicrobials found in the stomach and small intestine select for bacteria belonging to phyla Bacillus or Proteobacteria.[41,62] Microbes found in the stomach and small intestine are exposed to differing and changing conditions (e.g. diverse pH range that changes from acidic to neutral over its length, low digesta residence times etc.) and the host digestion and absorption of nutrients which likely combine to limit microbial growth to lower densities.[63,64]

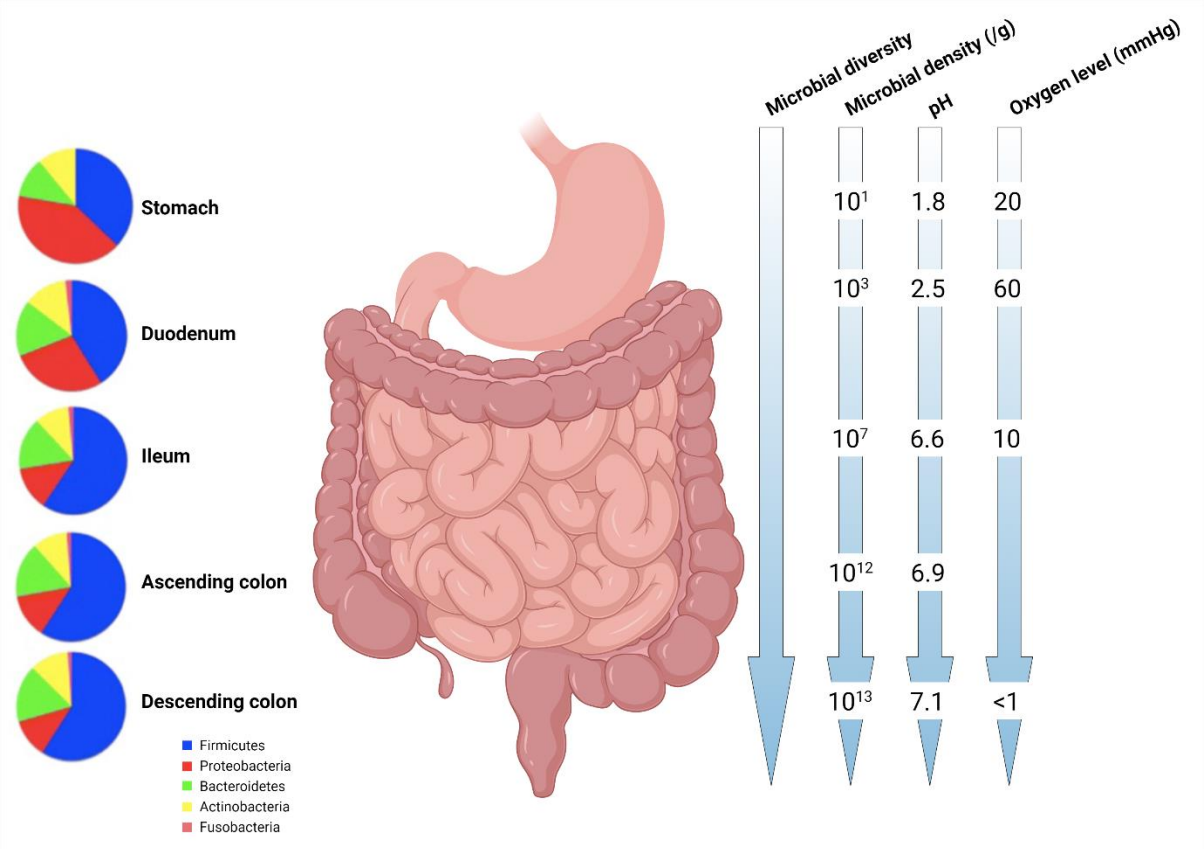

Figure 2. Compositional changes of the microbiota along the gastrointestinal tract. Figure adapted from Mailhe et al. (2018)[62] and created with BioRender.com.

By contrast, strictly anaerobic bacteria belonging to the phylum Firmicutes are abundant in the large intestine, where pH is higher and less oxygen is available (Figure 2).[62,65,66] The microbiota composition also differs by anatomical location within the colon, with differing proportions of bacteria from the Firmicutes phylum, *Bacteroides* genus and Deferribacteraceae family.[67] Diversity of bacteria has also been shown to differ between the lumen and inter-fold regions of the intestines. For example, an abundance of bacteria from the *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae* families can be found in the lumen, compared with a relatively higher abundance of bacteria from the *Lachnospiraceae* and *Ruminococcaceae* families in the inter-fold regions.[41] Furthermore, the greater amounts of mucus found in the inter-fold regions may serve as an additional nutrient source, allowing for colonisation of greater varieties of microbes.[68] The caecum and colon harbour the densest and most diverse microbial communities due to a more neutral pH, lower concentrations of antimicrobials and slower transit time.[41] In addition, the lower availability of simple carbon sources selects for the growth of fermentative polysaccharide-degrading anaerobes such as *Bacteroidaceae* and *Clostridiaceae* families.[41]

1.2.3. Functional roles

1.2.3.1. Immunological and protective effects

In utero, the foetal immune system must be tolerant to maternal alloantigens. Prior to birth and at approximately 13 weeks of gestation, antibodies (primarily immunoglobulin (Ig) G) generated by the maternal immune system are transferred to the foetus via the placenta. However, the majority is transferred towards the end of the third trimester.[33,69] However, at birth, a rapid change in immune responses is required to combat, reconcile and become tolerant to the sudden exposure to environmental antigens, the majority of which is derived from the intestinal microbiota.[70] Infants born prematurely have lower levels of antibodies and are more susceptible to infection.

Following birth, breast milk provides infants with additional protection in antibodies (primarily IgA) and other innate immune factors, such as chemokines and cytokines. This observation is relevant for

colostrum, and over time, the components of breast milk change from a primarily protective role to a nutritional role.[71-73]

The maturation of the immune system requires an intestinal microbiota, the absence of which impacts all aspects of the immune system. Improper development brings about several immunological defects, including increased susceptibility to infections and altered immune homeostasis.[74-76] Training of the infant immune system is also required for optimal intestinal function, including vascular supply,[77] epithelial healing,[78] nutrient absorption, and protection from infection.[79] For example, appropriate adaptive immune responses are required for lipid absorption and metabolism.[79,80] Shulzhenko *et al.* identified inter-connecting regulatory signalling networks which balance metabolism and the innate defensive mechanisms in small intestinal epithelial cells.[79] If IgA concentrations in intestinal digesta are altered, these networks become unbalanced and may cause irregular upregulation of certain pathways (e.g. innate immunity), downregulating others (e.g. fat uptake and metabolism).[79]

Invariant natural killer T cells are fundamentally important to the development of adaptive immune responses and are activated to combat a number of infections and inflammatory diseases.[81,82] The accumulation of these cells in body tissues contrasts starkly between GF and conventionally reared rodents, with cell numbers decreased in peripheral tissues (such as the spleen or liver) but increased in mucosal tissues (such as lung and colon) in GF mice.[83] Similarly, GF animals display altered invariant natural killer T cells' responsiveness to antigen stimulation, which also differs between the sites of the body, with hypo- and hyper-responsiveness at peripheral or mucosal sites, respectively.[84,85] This exaggerated accumulation, accompanied by hyper-responsiveness, is associated with augmented inflammatory responses that may induce colitis.[84,85] This effect was normalised following colonisation with a conventional microbiota within two weeks of life.[84,85]

Immunoglobulins are an integral part of the immune response due to their ability to specifically recognise and bind to antigens, facilitating antigen destruction. There are five main classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE), characterised by the type of heavy chain within their

structure, resulting in differences in their function and type of immune response elicited by each molecule.

A study by Cahenzli *et al.* revealed that IgE levels in GF mice were drastically increased compared with conventionally reared mice and suggested that IgE levels are regulated by the intestinal microbiota.[86] Elevated IgE levels led to an exaggerated sensitivity to anaphylaxis in mice, but these effects were reversed following colonisation with a conventional microbiota within four weeks of postnatal life but not thereafter.[86] It was also noted that microbial diversity rather than colonisation with specific microbial species affected IgE production, and low microbial diversity was insufficient to normalise IgE levels during early postnatal life.[86] Additionally, it has been demonstrated that the 'allergy phenotype' is transferrable via transplantation of the faecal microbiota in GF mice which are inherently susceptible to anaphylactic responses to food quantified by a drop in body temperature. The colonisation of these mice with the faecal microbiota from healthy infants protected the mice from anaphylactic responses, but not when colonised using the faecal microbiota from infants allergic to bovine milk.[87]

1.2.3.2. Gastrointestinal morphology

Microbe-microbe and microbe-host interactions are essential for developing and sustaining homeostasis of the intestinal epithelial cell layer.[6] The rates of epithelial cell proliferation and death must be balanced for the normal barrier function of the GIT, and it has been demonstrated that the microbiota is crucial for maintaining this balance.[88] Commensal microbes can promote cell renewal and repair[89] and are suggested to be essential for the induction of inflammation.[88,90,91] The metabolic capabilities that the intestinal microbiota exhibits, for example, organic acid production, play a role in regulating epithelial cell growth and differentiation.[92]

The microbiota also plays a role in the maintenance of epithelial integrity. Research in this area has primarily focused on developing probiotics to improve or repair barrier function. Probiotics are defined as "living microorganisms, which, when administered in adequate amounts, confer health

benefits on the host" by the Food and Agricultural Organization of the United Nations and the World Health Organisation.[93] For example, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Bacteroides thetaiotaomicron* reduced inflammation, repaired barrier integrity, helped prevent the onset of structural injury and modulated expression of genes required for intestinal barrier function.[94-97]

Furthermore, the microbiota aids in the maintenance of the mucus layer, which is a physical barrier that protects the host from continuous immune stimulation and subsequent inflammation.[33] GF rodents are observed to have a thinner mucus layer when compared with conventional rodents.[98-104] However, this effect was reversed following treatment with microbial bacterial products such as lipopolysaccharides and peptidoglycan.[102] Some species have been demonstrated to directly affect the mucus layer. For example, the mucin-degrading bacterium *Akkermansia muciniphila* has been shown to increase the thickness of the mucus layer in mice fed a high-fat diet.[105] The degradation of the mucus layer by that bacterium encourages the host production of mucin, further feeding it and forming a positive feedback loop.

1.2.3.3. Digestion and metabolism

The intestinal microbiota assists the host in digestion and metabolism and utilises nutrients that may otherwise be non-digestible by the host alone.[106] The microbiota may directly or indirectly influence host metabolism, for example, directly breaking down foods as they progress through the GIT, altering gene expression of host cells to facilitate the breakdown of foods, and providing enzymes that are not encoded in the human genome.[6,107] Most notably, the microbiota plays important roles in carbohydrate,[108,109] lipid[110] and protein metabolism,[111] and vitamin synthesis,[112] as summarised below.

Carbohydrate metabolism

Mammals are limited in their ability to degrade and utilise polysaccharides and so a large proportion of undigested dietary carbohydrates passes into the large intestine.[106] Microbes in the large intestine

rely on these fermentable carbon sources and produce key products, including organic acids and gases.[107]

Microbes also produce intermediate fermentation products, which are then used by other microbes.[107] This interaction is termed microbial cross-feeding. Examples of intermediate products include fumarate, succinate, and lactate.[113] Microbial co-culture studies have been used to demonstrate the reliance certain microbes may have on the products produced by others. For example, it has been observed that *Eubacterium hallii* cannot grow without the presence of lactate-producing bacteria from the *Bifidobacterium* genus.[114]

Lipid metabolism

The intestinal microbiota has been observed to indirectly affect lipid metabolism through its production of intermediate precursors, which are then further metabolised by the host to generate compounds that modulate lipid metabolism.[115,116] For example, the resident microbiota produce trimethylamine following the metabolism of choline or phosphatidylcholine, which is converted into trimethylamine N-oxide by the host.[115,116] This end-product has been linked to the progression of atherosclerotic disease.[115] Other microbial metabolites have similarly been observed to affect other metabolic processes, including hepatic cholesterol, sterol, glucose and insulin metabolism.[110,117]

Protein metabolism

The proteolytic capability of the colonic microbiota has been extensively reviewed.[107] Proteolytic microbes metabolise dietary and endogenous proteins and convert them into shorter peptides, amino acids, organic acids and gases.[111] This activity is critically important as these metabolites can affect the host metabolism and the immune and nervous systems.[118]

Human cells cannot synthesise some amino acids and rely on dietary intake and microbial amino acid synthesis to produce these essential molecules, such as tryptophan.[119,120] *Bacteroides* and *Propionibacterium* were identified as the predominant proteolytic bacterial genera, including

Clostridia, *Streptococcus*, *Staphylococcus* and *Bacillus*.[111] Excessive consumption of protein can lead to more undigested proteins or protein residues reaching the colon, providing substrates for microbes that can metabolise peptides[121,122]. The degradation products elevate luminal pH, which favours pathogen proliferation and may lead to adverse health effects.[121,122]

Vitamin synthesis

The intestinal microbiota also synthesises vitamins, symbiotically providing for host health.[106] When comparing GF and conventionally-reared rodents, GF rodents require more vitamins, for example, K and B (B_{12}, biotin, folic acid, cobalamin, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine) in their diet.[112,123-125] A lack of these essential vitamins has downstream effects on host health and metabolism.[126]

1.2.4. Factors affecting infant microbial composition and function

Development and maturation of the GIT microbiota are first shaped in early postnatal life, and the microbial signature stabilises at around three years of age. Its composition is affected by factors such as gestational age, mode of delivery, feeding methods and weaning period.[33]

1.2.4.1. Birth gestational age

Infant gestational age at birth is a major determining factor of GIT microbial colonisation, and a stark contrast is evident between preterm (<37 gestational weeks) and full-term infants. This difference is attributed to microbial colonisation challenges due to organ immaturity,[127] and environmental factors such as antibiotic use,[31] hospital stay[128] and enteral feeding.[127] The intestinal microbiota composition of preterm infants tends to have lower diversity, accompanied by increased colonisation by Proteobacteria phylum[129] and lower levels of strict anaerobes from the *Bifidobacteriaceae*,[130] *Bacteroidaceae*[131] and *Coriobacteriaceae* families.[128]

Microbiota composition of maternal breast milk has also been shown to differ between full- and preterm infants,[132] however, its contribution to microbial colonisation and the subsequent effect on

neonatal intestinal defences are not yet understood. Preterm birth is also associated with neonatal necrotising enterocolitis,[129] which has been linked to high levels of Proteobacteria being predictive of the disease.[133]

1.2.4.2. Mode of delivery

Delivery of the infant via C-section disrupts the first phase of colonisation as the infant is exposed to other sources, such as the maternal skin and hospital environment, rather than vaginal microbes.[129,134] Jakobsson *et al.* demonstrated that vaginally-delivered infants shared a large proportion of microbial 16S rRNA gene sequences with their mother than with other mothers for up to two years of age, particularly within bacteria from the Bacteroidetes and Firmicutes phyla.[135] A lower proportion of these bacterial phyla was found for infants delivered via C-section.[135]

It has also been shown that C-section delivery delays colonisation of the infant GIT,[135] leading to decreased overall microbial diversity, when compared with vaginal delivery.[129] This difference in microbiota composition between C-section or vaginally born infants has also been detected in faecal samples at seven years of age.[136] This observation suggests that following disruption of the initial colonisation of the microbiota, it may be unable to correct itself over time. C-section birth has been associated with increasing risk of chronic immune disorders, including but not limited to asthma, juvenile arthritis, inflammatory bowel disease and obesity.[31,137,138]

1.2.4.3. Feeding methods

Optimal colonisation of the infant GIT is promoted by exclusive breastfeeding for the first four to six months due to nutrients and other factors present in breast milk which encourage the proliferation of 'key' bacteria that direct the immune system to favour immune homeostasis over inflammation.[134,139] Such bacteria, including *Bacteroides fragilis*, *Bifidobacteria infantis* and *Lactobacillus acidophilus*, stimulate endogenous production of secretory IgA, activation of regulatory T cells and anti-inflammatory molecules – all necessary to encourage host homeostasis.[34,140,141] Lactoferrin, known for its antimicrobial, immuno-stimulatory and immuno-modulatory properties, is an important factor in

the initiation, development and composition of the infant microbiota and encourages the proliferation of beneficial bacteria from *Lactobacillaceae* and *Bifidobacteriaceae* families.[142]

In addition, breast milk has been demonstrated to provide passive protection and stimulate the development of the infant's immune system.[143] It contains polymeric IgA and defensins, which can interfere with pathogen attachment and uptake[141], omega-3 fatty acids[144] and transforming growth factor-β[145] which can activate enterocytes to cause anti-inflammation, and lactoferrin which can interact with the GIT and promote immune homeostasis.[134,141] Furthermore, Penders *et al.* have showed that exclusively formula-fed infants are more often colonised by bacteria such as *Escherichia coli*, *Clostridium difficile*, *B. fragilis,* lactobacilli, enterococci and enterobacteria when compared to exclusively breastfed infants.[146-149] The increased abundance of *Clostridium* spp. in formula-fed infants has been implicated in the increased incidence of atopy in later life.[150]

Human milk oligosaccharides (HMOs) are responsible for selectively promoting the growth and function of beneficial bacteria.[46] As infants lack the necessary enzymes to digest HMOs, they reach the large intestine. There, they function as a prebiotic,[23,151] promoting and stimulating the growth of specific genera such as *Staphylococcus*,[152] *Bifidobacterium*,[153] *Streptococcus*, *Lactobacillus*[154] and *Bacteroides*.[155] Only some bacteria (such as *Bifidobacterium longum* subspecies *infantis* lineage) harbour genes to express all enzymes required for degrading and utilising the entire HMO molecule.[46] However, other bacteria may cleave and utilise specific components of the HMO molecule.[46] A two-fold increase of faecal *Bifidobacterium* is explained by HMOs in breastfed infants compared to formula-fed infants.[31]

1.2.4.4. Weaning period

The introduction of solid foods and the progressive reduction of milk feeding lead to major intestinal microbiota compositional and functional changes. Bacteria belonging to genera *Bifidobacterium*, *Blautia* and *Bacteroides* are most abundant after weaning,[156] and the compositional differences between breastfed or formula-fed infants slowly decrease.[31,142,157]

The sudden change from high-fat, low-carbohydrate milk (pre-weaning) to high-carbohydrate, low-fat food (weaning and onward) results in a stark change of available nutrients to the commensal microbes.[158] The predominant genera post-weaning are microbes efficient at degrading dietary fibres and producing organic fatty acids.[159] This results in a microbiota composition more indicative of an adult-like microbiota.[158-160]

A study by Al Nabhani *et al.* examined the importance of the weaning period in further developing the immune system using GF and specific-pathogen-free mouse models.[161] The shift in microbiota composition during weaning induced the expression of immune cytokines such as tumour necrosis factor-α and interferon-γ.[161] The authors termed this effect a 'weaning reaction,' which was not observed in the GF mice as microbiota-induced Treg cells were deemed necessary.[161] Perturbances to the intestinal microbiota pre-weaning or exposure to a conventional microbiota post-weaning negatively affect this 'weaning reaction,' which may lead to increased susceptibility to colitis or allergic inflammation later in life.

1.3. Infant formulae

Breast milk is considered the optimal diet for infants, with the World Health Organisation recommending infants be exclusively breastfed for the first six months of postnatal life.[162-164] Extensive evidence has shown that breast milk contains a variety of bioactive agents that modify the function of the GIT and the immune system, as well as brain development.[162,165] However, breastfeeding may not always be possible. Infant formulae are industrially produced substitutes for breast milk consumption, which attempts to mimic the nutritional composition of breast milk as closely as possible, typically based on the properties of bovine milk.[166]

Infant formulae must include proper amounts of water, carbohydrates, proteins, lipids, vitamins, and minerals that meet all the requirements of normal physical growth.[167] As there is no mechanical breakdown in infants' digestion during the first 6 months of postnatal life, there must also be sufficient amounts of protein in a form that infants can use can be easily broken down chemically.[167,168] Excess

protein may lead to increased abdominal cramps and undigested residue in the colon, leading to colic-associated symptoms.[168]

The nutritional composition of infant formulae is strictly regulated, and each manufacturer must follow established guidelines set by their respective government agencies. For instance, all the major components added to formulae (proteins, lipids, carbohydrates) have a range of minimum and maximum values for their effectiveness. These components must also have an established history of safe use and remain stable throughout the shelf life of the product.[167,169,170]

Milk digestion begins in the mouth, where the slightly acidic saliva combines with the milk and begins to break it down. It then moves to the acidic environment of the stomach, where pepsin starts protein digestion by breaking down milk proteins into peptides. These smaller fragments then move into the small intestine, where proteases and lipases, produced by the pancreas, further break down milk proteins and lipids (and their fragments). Bile is also secreted into the small intestine to help with lipid digestion. Milk proteins become smaller peptides and amino acids, and milk fats become diglycerides, monoglycerides and fatty acids. Lactase is secreted by the host intestinal epithelial cells and hydrolyses lactose to its constituent monosaccharides, glucose, and galactose. Monosaccharides, peptides, amino acids, and micronutrients (e.g., calcium) are absorbed by the enterocytes of the small intestine into the hepatic portal vein. The liver regulates the distribution of these blood-borne nutrients to the rest of the body. Any undigested components, for example any undigested lactose, pass into the large intestine, to be further broken down by both the host cells and the resident microbiota.

Milk from domesticated ruminant species, in particular bovine milk, have been used as the basis for infant formulae.[167] However, bovine milk is one of the most common causes of food allergy (5-7% in formula-fed infants, 0.5-1% in breastfed infants).[167,171,172] The clinical manifestations of bovine milk allergy vary widely in type and severity and are presented in the first year of life.[173] As such, there has been increasing research into developing infant milk formulae from caprine, ovine, equine and

camelid species. These, in particular caprine or ovine milk, have been marketed as substitutes for bovine milk in managing bovine milk allergy in infants and children.[167]

1.3.1. Milk composition

The composition of the various components in milk evolved to meet the nutritional and physiological needs of their specific species' neonates.[174] This variation is reflected in differences between breast milk and bovine, caprine, and ovine milk used to make infant formulae (Table 1). It is also worth noting that genetic, physiological, and nutritional factors and environmental conditions affect the composition of milk. Milk is composed of water, proteins (casein and whey), lipids, carbohydrates (lactose, oligosaccharides), and minerals (ash). The compositional differences between breast milk and bovine, caprine, and ovine milk may result in downstream differences in infant development and health.

Table 1. Chemical concentration of human, bovine, caprine and ovine milk.[175]

Properties (%)	Human	Bovine	Caprine	Ovine
Protein	1.2	3.5	3.6	5.8
Casein	0.5	2.8	2.7	4.9
Whey	0.7	0.7	0.9	0.9
Lipid	3.8	3.7	4.1	7.9
Carbohydrate	7.0	4.8	4.7	4.5
Ash	0.2	0.7	0.8	0.8

Proteins

The protein concentration is lower in breast milk than in ruminant milk, with ovine milk having the highest concentration (Table 1). Furthermore, the continued prevalence of the apparent intolerance to bovine milk has increased interest in formulae made up of caprine or ovine milk.[166] Intolerance to caprine or ovine milk has not been observed, likely due to differences in compositional and physicochemical characteristics compared to bovine milk.[176,177] Differing beta- and alpha-casein protein ratios influence the casein micelle formation with higher mineralisation and lower colloidal

stability, resulting in faster coagulation and delayed gastric emptying.[178-180] Furthermore, amino acid sequence differences in milk proteins may result in different peptide formations during digestion.[181,182]

Lipids

The lipid component is the most variable among bovine, caprine, and ovine milk, with ovine milk having the highest concentration and milk from bovine and caprine species having a similar concentration to human breast milk (Table 1). Lipids are the main source of energy and building blocks for tissue growth during infant development.[183,184] Fatty acids (specifically linoleic acid) are the precursors of biologically active substances and are essential for the proper development of the newborn. The compositional differences in fatty acids found between human, bovine, ovine and caprine milk are shown in Table 2. Approximately 98% of lipids in milk are triacylglycerols, and the remaining lipids (2%) are composed of phospholipids, cholesterol, carotenoids, fat-soluble vitamins, some fatty acids, and cholesterol esters.[185,186]

Table 2. Fatty acids composition found in human, bovine, caprine and ovine milk.[187,188]

Fatty acid		Composition (%)			
		Human	Bovine	Caprine	Ovine
4:0	Butyric acid		3.88	2.64	2.18
6:0	Caproic acid		2.49	2.11	2.39
8:0	Caprylic acid	0.22	1.39	2.41	2.73
10:0	Capric acid	1.63	3.05	9.35	9.97
12:0	Lauric acid	5.27	4.16	5.35	5.00
14:0	Myristic acid	5.76	11.40	12.00	9.81
14:1 *cis*-9	Myristoleic acid	0.44	1.11	0.24	0.18
16:0	Palmitic acid	21.36	29.40	27.50	28.20
16:1 *cis*-9	Palmitoleic acid	2.39	1.94	0.76	1.43
18:0	Stearic acid	6.92	11.40	6.92	8.88
18:1 *cis*-9	Oleic acid	38.57	21.90	16.40	17.20
18:2 *cis*-9, *cis*-12	Linoleic acid	13.34	1.94	1.99	3.19
18:3 *cis*-9, *cis*-12, *cis*-15	Linolenic acid	0.12	0.55	0.96	0.42
Saturated		42.13	70.10	74.70	72.40
Monounsaturated		42.29	25.80	20.40	22.00
Polyunsaturated		15.58	3.32	2.93	4.31

Carbohydrates

Breast milk has the highest concentration of carbohydrates compared to bovine, caprine, and ovine milk, which have similar concentrations (Table 1). Milk contains a large variety of complex oligosaccharides, which are typically composed of three to ten monosaccharide units, which include: glucose, galactose, N-acetyl-glucosamine, fucose and sialic acids.[189,190] Their biological activity is dependent on their structure. Human breast milk is a rich source of oligosaccharides, and more than 200 structures have been identified.[191,192] Less is known about ruminant milk oligosaccharides as their concentration is very low when compared with human breast milk. Through advanced analytical techniques, however, structural libraries of more than 50 bovine milk oligosaccharides,[193-195] 29 porcine milk oligosaccharides,[196] 20 caprine milk oligosaccharides,[197] and 12 camelid milk oligosaccharides[198] have been established. Only few oligosaccharide structures have been established for equine and ovine milk.[199-201] Furthermore, in comparison with human breast milk, the content of fucosylated oligosaccharides is comparatively low in the milk of domestic ruminant species.[199,201]

A comparative analysis of the oligosaccharide structures of bovine, caprine, ovine, porcine, equine and dromedary camel milk by Albrecht *et al.* found not only many similarities but also species-specific characteristics.[189] Sialylated oligosaccharides account for approximately 80%–90% of the total pool from the milk of all domestic ruminants.[189,202] Milk of grazing cows contains higher concentrations of sialic acid compared to non-grazing cows.[203] Milk oligosaccharides from camel milk were the most diverse, which included seven fucosylated structures. On the same note, porcine milk contained the highest percentage of neutral oligosaccharides and the most abundant variety of monosialylated and disialylated large oligosaccharides. Furthermore, the structures of porcine milk oligosaccharides were most similar to HMOs as compared with the other domestic ruminant species analysed.[189] This observation reinforces the hypothesis that milk oligosaccharides are tailored for the postnatal development of the GIT and that these molecules aid in preparation for certain post-weaning diets by acting as early dietary fibres.

Humans and pigs are monogastric and, therefore, have large intestinal fermenters, whereas cows, goats and sheep are ruminants; camels have a three-chambered digestive system and horses have an extended caecal digestive system. Furthermore, humans and pigs are omnivores, whereas all other species are herbivores. Pigs are considered to be excellent models for human large intestinal fibre metabolism, and the similarity between the human and porcine milk oligosaccharides highlights their usefulness in studying early-postnatal life nutritional programming and development of the GIT.[189]

1.4. Future perspectives

As summarised in this literature review, diet and nutrition play a crucial role in shaping the GIT microbiota, which has downstream effects on host health and development. Bovine milk has been a common basis for the development of infant formulae, but with the frequency of bovine milk allergy, increased attention has been given to other ruminant milk. The research undertaken in this Master's thesis addresses this, by aiming to understand the effects of consuming bovine, caprine or ovine milk on the composition and predictive function of the ileal and colonic microbiota in pigs as a model of the human infant. It was also hypothesised that the predictive function of the ileal and colonic microbiota would be similar between the three milk treatments due to a 'core functional' microbiota, whereby other microbes can undertake the function of other members in the microbial community.[55,60]

2. Effects of consuming bovine, caprine or ovine milk on ileal and colonic microbiota composition and functional potential

2.1. Abstract

This study aimed to understand the effects of bovine, ovine or caprine milk on the composition and functional potential of the ileal and colonic microbiota in pigs as a model of the human infant. Pigs at postnatal days 7-8 were fed exclusively milk for two weeks. Digesta samples were collected on postnatal days 21-22. Microbial DNA was extracted from digesta, and shotgun sequencing was performed to provide insight into microbiota composition and functional (as inferred from the gene abundance data) changes. Statistical significance was carried out using a statistical framework, 'analysis of the composition of microbiomes with bias correction'. Overall, there was no significant difference in the composition of the ileal or colonic microbiota at the higher taxonomic levels following feeding with the various milk formulae. However, the milk treatments did differentially affect taxa from *Desulfovibrio*, *Blautia*, *Ruminococcus* and *Prevotella* genera in the terminal ileum and taxa from *Selenomonas*, *Elusimicrobium* and *Salmonella* genera in the proximal colon. Some cluster analyses revealed similarities between the bovine and caprine groups, but limitations in the study reduced the confidence in these results. The functional potential of the ileal and colonic microbiota was not significantly different between diets. Some limitations of the experimental design were small sample sizes, a measurement of a single timepoint (snapshot), and the adjustment of milk volume to make protein concentration equal.

2.2. Introduction

Early postnatal life nutrition plays a significant role in shaping the development of the infant, and expectant mothers are commonly counselled that "breast is best" for their infant.[165] Beyond somatic growth, breast milk as a biological fluid has various other benefits, including modulation of postnatal gastrointestinal tract (GIT) function, immune ontogeny, and brain development.[167] As discussed in Section 1.2.4, diet plays an important role in shaping the composition of the GIT microbiota, with downstream effects on host development. Optimal colonisation of the infant GIT is thought to be

promoted by exclusive breastfeeding for the first four to six months due to nutrients and other factors present in breast milk which encourage the proliferation of 'key' microbes that direct the immune system to favour immune homeostasis over inflammation.[134,139] In addition, breast milk has been demonstrated to provide passive protection and stimulate the development of the infant's immune system.[143]

Whilst breastfeeding is the optimal diet for infants; this is not always possible. Infant formulae are industrially produced substitutes for breast milk, which attempts to mimic the nutritional composition of breast milk as closely as possible, typically based on the properties of bovine milk.[166] Caregivers increasingly seek other milk, such as caprine or ovine milk, to feed infants, largely due to anecdotal evidence of advantages over bovine milk. Concentrations of the various macronutrients and oligosaccharide profiles found within bovine, caprine and ovine milk differ from one another.[175] These compositional differences might result in differences in milk-derived substrates reaching the large intestine and available to the resident microbiota. However, the impact of consuming formulae made up of bovine, caprine or ovine milk on the composition of the GIT microbiota in early postnatal life has received limited attention.

Comparing the compositions as detailed in Table 1 of Section 1.3.1, it was hypothesised that the terminal ileal and proximal colonic microbiota of pigs fed ovine milk would harbour a different and more diverse composition than that of pigs fed bovine or caprine milk, as bovine and caprine milk are more similar in composition than ovine milk. It was also hypothesised that the predictive function of the terminal ileal and proximal colonic microbiota would be similar between the three milk treatments due to a 'core functional' microbiota, whereby other microbes can undertake the function of other members in the microbial community.[55,60]

2.3. Materials and Methods

The study was designed and undertaken by Dr Debashree Roy, a former PhD student at the Riddet Institute, hosted by Massey University, to investigate structural and physicochemical changes in milk

during gastric digestion using the bottle-fed pigs as an animal model of the human infant.[175] Additional ileal and colonic content samples were collected to address the aim and hypotheses of this Master thesis. I was involved in the study, from feeding the pigs to the sample collection.

All procedures involving animals were approved by the Massey University Animal Ethics Committee (MUAEC protocol no. 18/97). The pigs were transported from a commercial farm in Whanganui to the Massey Animal Research Facilities. Upon arrival, the pigs were weighed, received ear tags, and housed individually in cages. The pigs were examined daily for general health, well-being, and body temperature.

2.3.1. Milk preparation

Spray-dried bovine, caprine and ovine whole milk powders were purchased from Davis Food Ingredients (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand) and Spring Sheep Milk Co. (Hamilton, New Zealand), respectively. In addition, raw pooled whole bovine, caprine and ovine milk batches were acquired from November 2018 to January 2019 from the Massey University No.4 dairy farm (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand) and Phoenix Goats (Palmerston North, New Zealand), and Neer Enterprises Ltd (Carterton, New Zealand), respectively. Spray-dried milk powders and whole milk were kept at 4°C until required. Prepared milk was warmed in hot water baths prior to being fed to the pigs through suckling throughout the study. The pigs were weighed weekly, and their daily ration was adjusted accordingly.

2.3.2. Experimental design

The experimental design of the study was described in Roy *et al.* (2022)[175] and summarised in Figure 3. Three dietary formulae were prepared with reconstituted spray-dried powders of bovine, ovine or caprine whole milk (Table 2). The pigs were randomly allocated to one of the three dietary formulae in a block design, with 78 entire male pigs aged 7 to 8 postnatal days (PND) divided into three blocks of 26 pigs. Twenty-four pigs per treatment were required for statistical power analyses. This estimate

was based on previous studies wherein kinetic parameters of curd formation or gastric and intestinal digestion (the primary outcomes of the study) have been monitored in pigs.[204,205] Two pigs per treatment were added to replace pigs that did not learn to bottle feed within the first six days of the study (acclimatisation period) or were otherwise unable to be included.

The pigs were fed formulae made with reconstituted spray-dried milk powders from PND 7-8 to PND 16-17. The feeding frequency was hourly from PND 7-8 to PND 12-13, and 17 meals were offered from 06:00 to 22:00. This step was done to acclimatise and train the pigs to be bottle-fed. From then onwards, the feeding frequency was gradually decreased and therefore, the amount of milk given at each meal increased. From PND 13-14 to PND 16-17, seven meals were offered every 2.5 hours, and from PND 17-18 to PND 18-19, five meals were offered every 3.5 hours.

From PND 19-20 to PND 21-22, the pigs were fed fresh pasteurised whole bovine, caprine or ovine milk every 3.5 hours as described above, according to their assigned treatment. This step was to ensure that they could adapt to changes in taste when they shifted from a reconstituted whole milk powder diet to a fresh pasteurised whole milk diet (Table 3). However, fresh milk was not fed from the beginning of the study due to the limited quantity of fresh pasteurised ovine and caprine milk from suppliers.

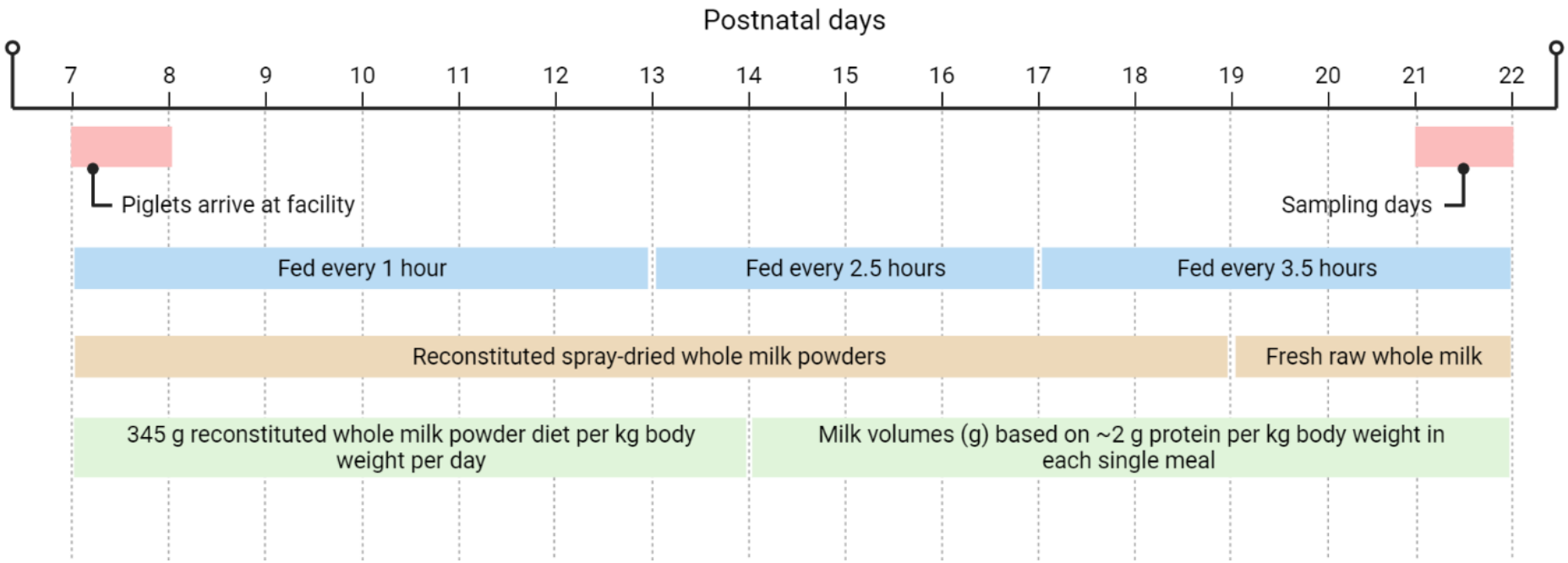

Figure 3. Experimental design timeline.

Furthermore, from PND 15, the volumes of milk were calculated to deliver constant amounts of protein, and as each ruminant milk naturally has different protein concentrations, the total volume of milk fed varied (Table 4). Protein concentrations were kept constant as the primary outcome of the study was to analyse curd formation in the digestion of the ruminant milk.[175]

On sampling day (PND 22), the pigs received one serving of their respective fresh pasteurised milk (bovine, caprine or ovine milk) at a set amount of protein per day (2 g/kg body weight) in the morning. The meals were offered at a set time to ensure samples were collected at the same time postprandially. The pigs consumed their meal within two to three minutes. The final calculated amount and composition of raw milk from each species received for their last meal are shown in Table 4.

Table 3. Type of formulae received and calculated volumes.[175]

Postnatal day	Type of formulae	Amount of formulae
7-14	Reconstituted whole milk diet with mineral and vitamin premix	345 g reconstituted whole milk powder diet per kg body weight per day
15-18	Reconstituted whole milk diet with mineral and vitamin premix	Milk volumes (g) based on ~2 g protein per kg body weight in each single meal
19-22	Fresh whole milk diet	Milk volumes (g) based on ~2 g protein per kg body weight in each single meal

Table 4. Calculated intake of raw milk from ovine, caprine and ovine species following normalising to 2 g protein per kg body weight in the last meal on the sampling day.[175]

Intake	Bovine milk	Caprine milk	Ovine milk
Fresh milk (g/kg BW)	55.3	63.1	31.9
Protein (g/kg BW)	2.0	2.0	2.0
Fat (g/kg BW)	2.2	2.0	2.0
Lactose (g/kg BW)	2.5	2.5	1.3
Dry matter (g/kg BW)	7.2	7.1	5.6
Gross energy (kcal/kg BW)	41.9	38.6	34.3

Half of the pigs were euthanised and sampled on PND 21 (n=13) and the remaining half on PND 22 (n=13) for logistics reasons due to the time required for collecting samples from each piglet (15

minutes). Four pigs from each treatment were euthanised at 0, 30, 90, 150 and 210 minutes after feeding. An additional four pigs per treatment received their last milk meal with titanium dioxide, an indigestible marker, to measure digesta flow, and were euthanised at 210 minutes after feeding. Each piglet was anaesthetised with Zoletil 100 (zolazepam and tiletamine, both at 50 mg/mL) and reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine, both at 100 mg/mL. It was administered at a dose rate of 0.4 mL of the mixed solution per 10 kg body weight by intramuscular injection. Immediately after sedation, the pigs were euthanised by an intracardial injection (0.3 mL/kg BW) of sodium pentobarbitone.

2.3.3. Sample collection

Following euthanasia, the abdomen of each animal was opened, and the entire stomach, jejunum, duodenum, terminal ileum, caecum, proximal large intestine, and distal large intestine were removed. Any blood was washed away using sterile deionised water. Each section was carefully divided and dried using absorbent paper towels. Digesta samples from these sections were collected and stored in microcentrifuge tubes containing RNA Later (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and temporarily kept on dry ice until the completion of the collection process. Samples were then stored in -80°C freezer until needed.

2.3.4. DNA extraction

Genomic DNA was extracted from ileal and proximal colonic digesta samples at the 210 minutes post-feeding timepoint. This timepoint was chosen as it offered the greatest number of samples as opposed to the other available timepoints. First, the samples were allowed to thaw on ice and then centrifuged at 10,000 x g for 5 minutes to pellet the solid sample. The supernatant was discarded, and the resultant pellet was then washed with 500 µL phosphate buffered solution to remove residual RNA Later. DNA extraction of the digesta samples was then performed using the Machery-Nagel NucleoSpin® Soil kit (Machery-Nagel, Düren, Germany) following the manufacturer's instructions with an additional

elution step, whereby the eluted flow-through was transferred back into the same column to repeat the elution process to increase the yield. The extracted DNA was stored at -80°C until required.

2.3.5. DNA purity and concentration

The purity and concentration of the DNA were determined using agarose gel electrophoresis and measuring the absorption of the DNA at 260 nm and 280 nm using the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). This measurement indicates any protein contamination, with 1.80 being the optimal ratio for DNA. The elution buffer from the DNA extraction kit was used as the blank. These measures were performed on the day of the extraction. Each test used 2 µL of the sample.

2.3.5.1. Agarose gel electrophoresis

A 50x buffer stock was prepared using the following compounds: 242 g of Tris-base (Sigma-Aldrich, Burlington, Massachusetts, USA), 57.1 mL of 100% acetic acid (Sigma-Aldrich, Burlington, Massachusetts, USA), 100 mL of 0.5M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Burlington, Massachusetts, USA), and dH_2O added to make up to 1 L tris-base, acetic acid and EDTA buffer. This buffer was then diluted to 1x concentration as needed.

1% agarose in tris-base, acetic acid and EDTA buffer containing SYBR-Safe (1 µL per 10 mL gel) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to visualise the extracted DNA. Each well contained 2 µL DNA, with 2 µL DNA marker (Invitrogen TrackIt 1 Kb Plus DNA Ladder) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) on both sides of the gel. The remainder of the well contents included 1 µL of Bluejuice (DNA loading dye) and 7 µL dH_2O. The gel electrophoresis conditions were 120 V for 30 to 45 minutes, then visualised, and an image was taken using the GELDOC (Bio-Rad Laboratories, Berkeley, California, USA). This data was used to estimate the size of the DNA extracted and to check for any unwanted fragmentation. A good, acceptable result is a smear from high to low molecular weight.

2.3.7. DNA sequencing

Metagenomic sequencing of the extracted DNA was performed by OSS Technology Hong Kong. The DNA also underwent quality control checks for purity and concentration by OSS Technology. DNA samples were then shotgun sequenced using the Illumina HiSeq platform with 2x150bp paired-end sequences.

Read pairs were aligned to the *Sus scrofa* reference genome Sscrofa11.1 (RefSeq: GCF_000003025.6) using the "mem" algorithm of BWA (V0.7.17-r1188).[206] Sequences mapped to the host genome were removed,[207] and the remaining reads were then paired using PEAR (V0.9.6).[208] Reads that were unable to be paired were pasted together using the "fuse" function from the BBMAP package version 38.22-0.[209] Finally, these paired reads from individual samples were compiled into a single sample file.

Metagenomic functions were obtained through the "blastx" program within Diamond (V0.9.22),[210] mapping the reads against the NCBI non-redundant database.[211] Megan (V6 Ultimate Edition) was used to assign putative functions to the alignment files produced by Diamond.[212] This alignment was performed by Paul Maclean (Statistician, AgResearch).

2.3.8. Statistical methods

All statistical analyses were performed by Dr Jane Mullaney, a co-supervisor at AgResearch, using the R packages vegan (V2.5-7) and ANCOMBC (V1.0.5).[213,214] Firstly, all reads were filtered to retain those classified as bacteria, and then the data were standardised to the mean (rarefaction). Taxa absent more than three times in at least 20% of the samples were removed to prevent any very low abundance taxa from skewing the results. For ANCOMBC, the data were also agglomerated to the genus level.

Alpha-diversity, with Chao1 and Shannon index values, was performed after filtering for bacteria but prior to standardising and filtering the low abundance data using one-way non-parametric ANOVA (analysis of variance) with Kruskal-Wallis testing and multiple comparison correction with Tukey

testing using Graphpad Prism V9.3.1 (471). Chao1 provides an estimate on species richness (total abundance), whereas the Shannon index quantifies species diversity (variety of taxa).

Beta-diversity comparing microbiotas across diets (bovine, caprine or ovine milk) was then examined. Several cluster analyses were performed to examine relationships between treatment groups. Firstly, principal component analysis (PCA) was used to assess the similarities between treatment groups. PCA reduces the dimensionality (variability) of large data sets by constructing principal components based on Euclidean distances and allows for easier data visualisation, interpretation and analyses.[215] Next, supervised analyses using sparse partial least squares discrimination analysis (sPLS-DA) plots were drawn to check the generalisation properties of the model and reduce dimensionality whilst being fully aware of the class labels.[216,217] Plot loadings derived from sPLS-DA identified the taxa responsible for the differences.[218]

Principal coordinate analysis (PCoA), using unifrac and Bray-Curtis measures, was compared with PCA to assess the similarities and dissimilarities between treatment groups.[219,220] PCoA is based on distances other than Euclidean distance and finds the potential principal components of the overall difference through dimensionality reduction.[221] Bray-Curtis dissimilarity quantifies the compositional dissimilarity between each sample.[222] Unifrac is another dissimilarity matrix, but unlike Bray-Curtis, it also incorporates phylogenetic distances between organisms in each sample.[219] Both PCA and PCoA yield plots contain the following: Axis 1 (x) represents the principal coordinate of the greatest variable; Axis 2 (y) represents the principal coordinate of the second greatest variable. Both axes also show the percentage of variables covered. The spatial distance between sample points within the plots represents the distance between samples. PCoA plots also include confidence ellipses (80%) and centroids for each treatment group.

From the PCA and PCoA analyses, plot loading barplots were drawn, showing which taxon may have had the largest impact on the principal components within their respective plots. The plot of the eigenvalues from their respective cluster analyses: Positive and negative values represent the positive

and inverse correlation between the effect each taxon has on the principal component, respectively. The magnitude of the value indicates the strength of this relationship.

There are some difficulties in performing statistics due to the high dimensionality, non-normality, and phylogenetic structure of microbiome data.[223] These issues are addressed using a non-parametric permutation ANOVA within 'analysis of the composition of microbiomes with bias correction' (ANCOMBC).[214,224] ANCOMBC estimates the unknown sampling fractions (when converting absolute abundance to relative abundance) and corrects bias introduced due to the differences among the samples. This is accomplished by testing the null hypothesis that the dispersion of each sample is equivalent to one another when measuring the distance from the centroid (it is, therefore, more accurate when n>5).[214] The '*p*' value is global (for all variables) and significant when $p<0.05$. The '*q*' value is the '*p*' value adjusted for the false discovery rate (ratio of the number of false positives to the number of total positive test results). For example, a *q* value of 0.01 means there is a 1% probability of a false positive result. The *'w'* value reports the number of times the null hypothesis was rejected (i.e., there was no statistical significance). Only *q* values <0.0001 and *w* values >20 were considered in this study.

Abundance (total and relative) stacked barplots were visualised using phyloseq,[225] and significantly different genera were graphed using Graphpad Prism V9.3.1 (471).

2.4. Results

2.4.1. Intake, growth, and health

All pigs sampled were healthy and ingested their milk quickly (within 2-3 minutes), indicating good growth and trained bottle feeding. All pigs that were sampled were in good health and included in the subsequent sample analyses in Section 2.4.2.

2.4.2. Samples used for analyses

Some pigs in each milk group did not have any digesta in the terminal ileal and proximal colonic segments available for collection, particularly from the ileum. The number of samples for each region (Ileum: target 4; Colon: target 8) for each treatment group is shown in Table 5.

Table 5. Breakdown of the number of terminal ileal and proximal colon digesta samples collected from the pigs fed bovine, caprine, or ovine milk sampled at 210 minutes post-feeding on postnatal day 22.

Treatment	Terminal Ileum	Proximal colon
Bovine milk	4	8
Ovine milk	4	7
Caprine milk	2	8
TOTAL	10	23

2.4.3. DNA purity and concentration

Nanodrop and agarose gel electrophoresis were performed to determine the purity and concentration of extracted DNA from terminal ileal (Table 6, Figure 4) and proximal colonic (Table 7, Figure 5) digesta before sending the samples for sequencing. These steps were repeated separately by OSS Technology Hong Kong prior to shotgun sequencing for quality check, and included in Appendix B. All samples from the terminal ileum and proximal colon passed the required quality checks and were thus sequenced.

Table 6. Purity and concentration of DNA extracted from samples of terminal ileum digesta collected from the pigs fed bovine, caprine, or ovine milk sampled at 210 minutes post-feeding on postnatal day 22.

Sample ID	Concentration (ng/µL)	A260 (10 mm)	A280 (10 mm)	A260/A280
Blank	0.000	0.000	0.000	0.000
3	15.150	0.301	0.152	1.968
6	46.950	0.941	0.507	1.859
7	43.500	0.870	0.469	1.855
14	145.300	2.901	1.555	1.863
25	7.550	0.151	0.083	1.819
42	28.950	0.575	0.318	1.798
48	31.550	0.725	0.458	1.734
50	10.200	0.202	0.129	1.557
53	39.100	0.786	0.452	1.746
55	226.450	4.536	2.414	1.882

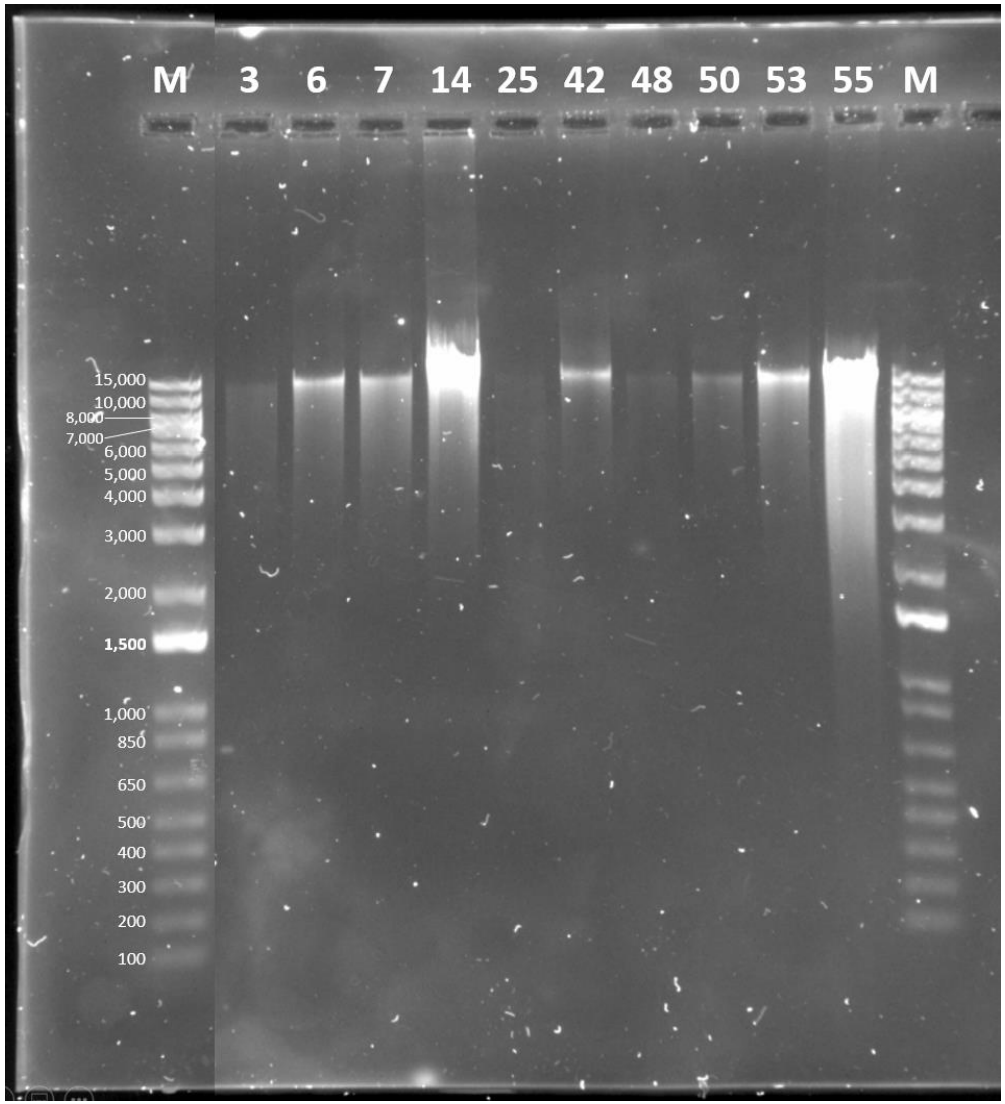

Figure 4. DNA visualised in 1% agarose gel of the samples from the terminal ileum digesta. Lanes are marked as follows: M: marker, the number corresponding to sample identification.

Table 7. Purity and concentration of DNA extracted from samples of proximal colon digesta collected from the pigs fed bovine, caprine, or ovine milk sampled at 210 minutes post-feeding on postnatal day 22.

Sample ID	Concentration (ng/µL)	A260 (10 mm)	A280 (10 mm)	A260/A280
Blank	0.0000	0.000	0.000	0.000
3	120.40	2.410	1.283	1.880
6	265.85	5.326	2.848	1.873
7	63.750	1.276	0.694	1.840
14	220.95	6.989	5.002	1.817
23	182.35	3.645	1.945	1.873
25	194.70	3.898	2.073	1.882
42	168.90	3.387	1.838	1.847
48	122.25	3.606	2.527	1.790
49	145.80	2.918	1.573	1.856
50	189.15	4.754	2.990	1.874
53	204.10	4.089	2.194	1.866
55	182.15	3.643	1.956	1.862
63	28.350	0.570	0.324	1.766
64	10.400	0.210	0.131	1.612
65	85.650	1.717	0.938	1.834
68	117.10	5.064	4.056	1.756
72	6.2500	0.129	0.088	1.488
73	6.6500	0.136	0.098	1.400
75	24.750	0.498	0.292	1.713
76	5.8000	0.116	0.086	1.349
78	21.000	0.425	0.247	1.736
80	16.150	0.669	0.568	1.455
83	33.350	0.669	0.385	1.742

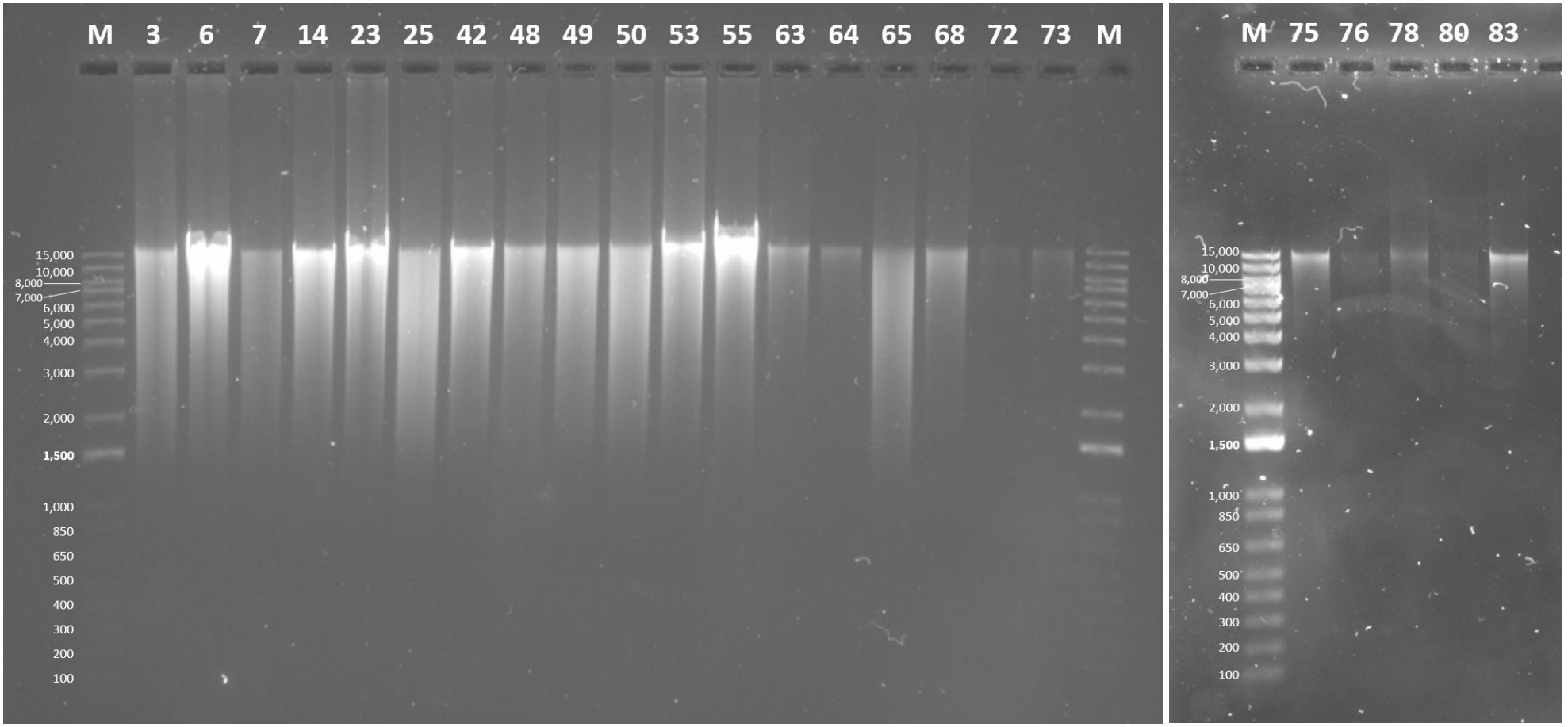

Figure 5. DNA visualised in 1% agarose gel of the samples from the proximal colon digesta. Lanes are marked as follows: M: marker, the number corresponding to sample identification.

2.4.4. Taxonomical composition

All the data represents samples collected at a single time point: 210 minutes post feeding on postnatal day 22 from pigs fed bovine, caprine, or ovine milk for two weeks.

Sequenced data quality, including the number of counts per sample, number of reads assigned, and coverage, was verified by Paul Mclean (Statistician, AgResearch) and attached in Appendix D. Overall the sequenced data quality was as expected.

Alpha-diversity was measured by CHAO1 (Figures 6A and 6B) and Shannon (Figures 6C and 6D) indexes. The CHAO1 index counts the number of unique species and considers the rare species present. The Shannon index measures how evenly distributed the diversity is in each sample, independent of species richness.[226]

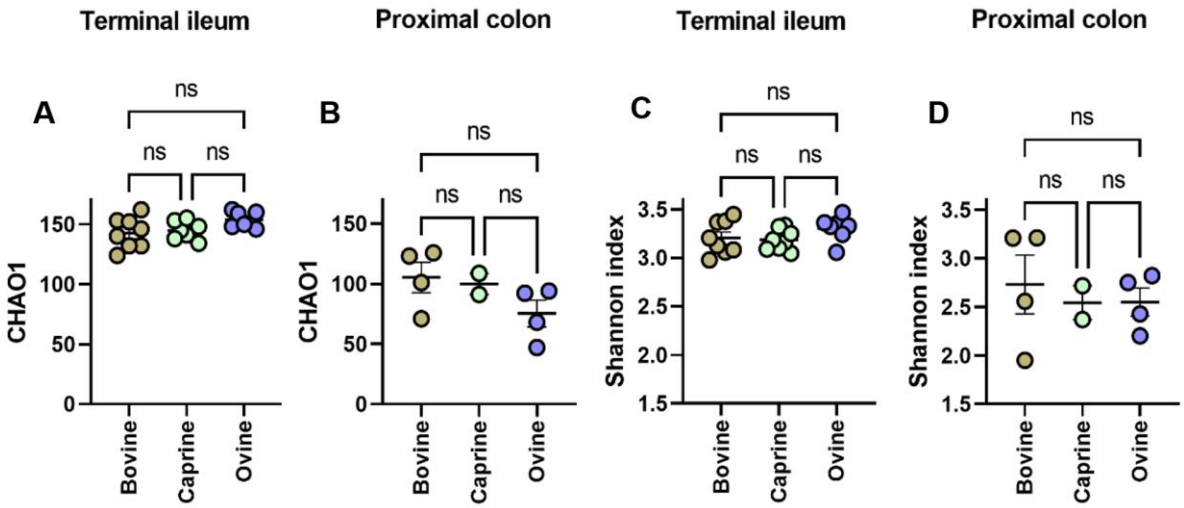

Figure 6. CHAO1 and Shannon diversity indexes of terminal ileal and proximal colonic microbiota of digesta samples as described previously. A: CHAO1: Terminal ileum; B: Proximal colon; C: Shannon index: Terminal ileum; D: Proximal colon. Key: brown – bovine; green – caprine; blue – ovine.

2.4.4.1. Terminal ileal microbiota

Firstly, the terminal ileum was examined (n=11). A PCA plot was drawn to examine the similarities between treatment groups (Figure 7). Samples belonging to pigs fed caprine milk form a distinct cluster, whereas an overlap was observed for clusters of samples belonging to pigs fed bovine or ovine

milk (Figure 7). A high percentage of explained variance (94%) within the plot gives confidence to these observations.

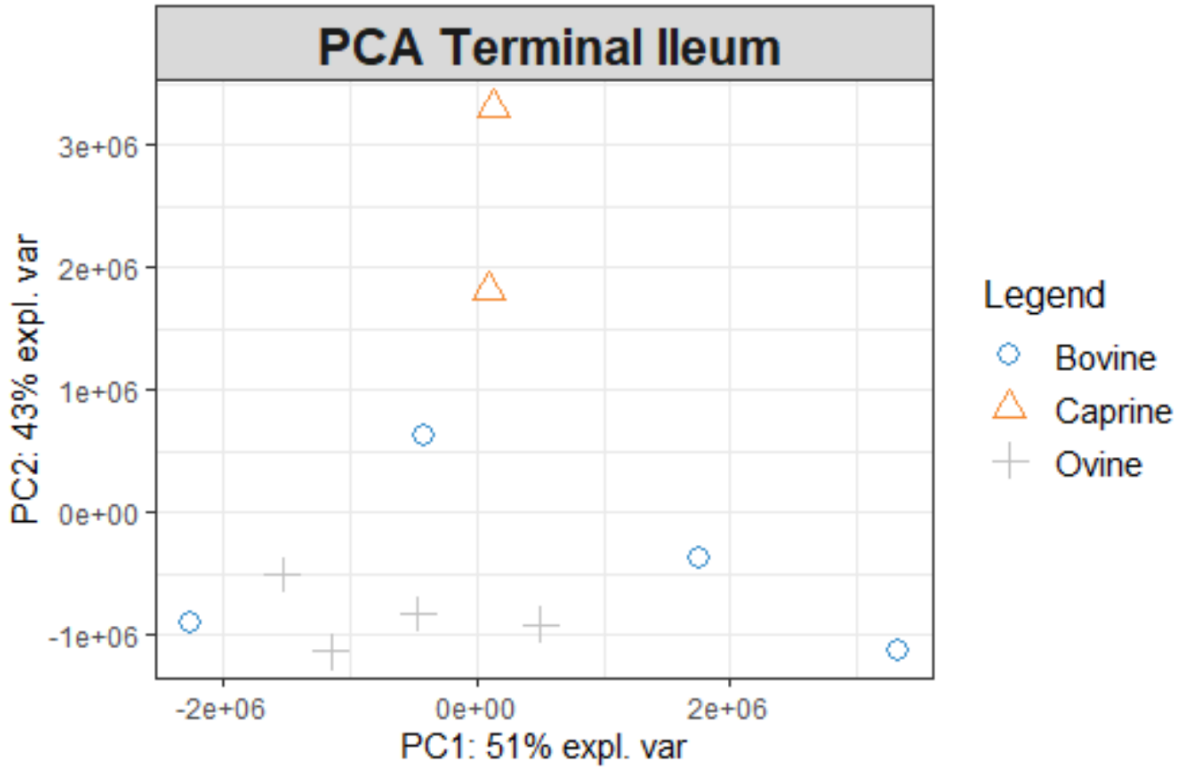

Figure 7. PCA plot of terminal ileal microbiota of digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine.

Plot loadings of principal components 1 (Figure 8A) and 2 (Figure 8B) were visualised in barplots. These plots show which taxon may have had the largest impact on the principal components within the PCA shown in Figure 7. Bacteria belonging to families *Pasteurellaceae* and *Enterobacteriaceae* had the largest effect on principal component 1 (Figure 8A). On the other hand, unclassified Gammaproteobacteria class, unclassified Enterobacterales order, and *Clostriodioides difficile*, *Streptococcus suis*, *Glaesserella parasuis* and *Actinobacillus indolicus* have a weak influence on principal component 1 (Figure 8A). *Clostridioides difficile* has the greatest influence on principal component 2 (Figure 8B). Bacteria belonging to families *Enterobacteriaceae* and *Pasteurellaceae* had some influence on principal component 2 (Figure 8B). All other taxa had minimal influence (Figure 8B).

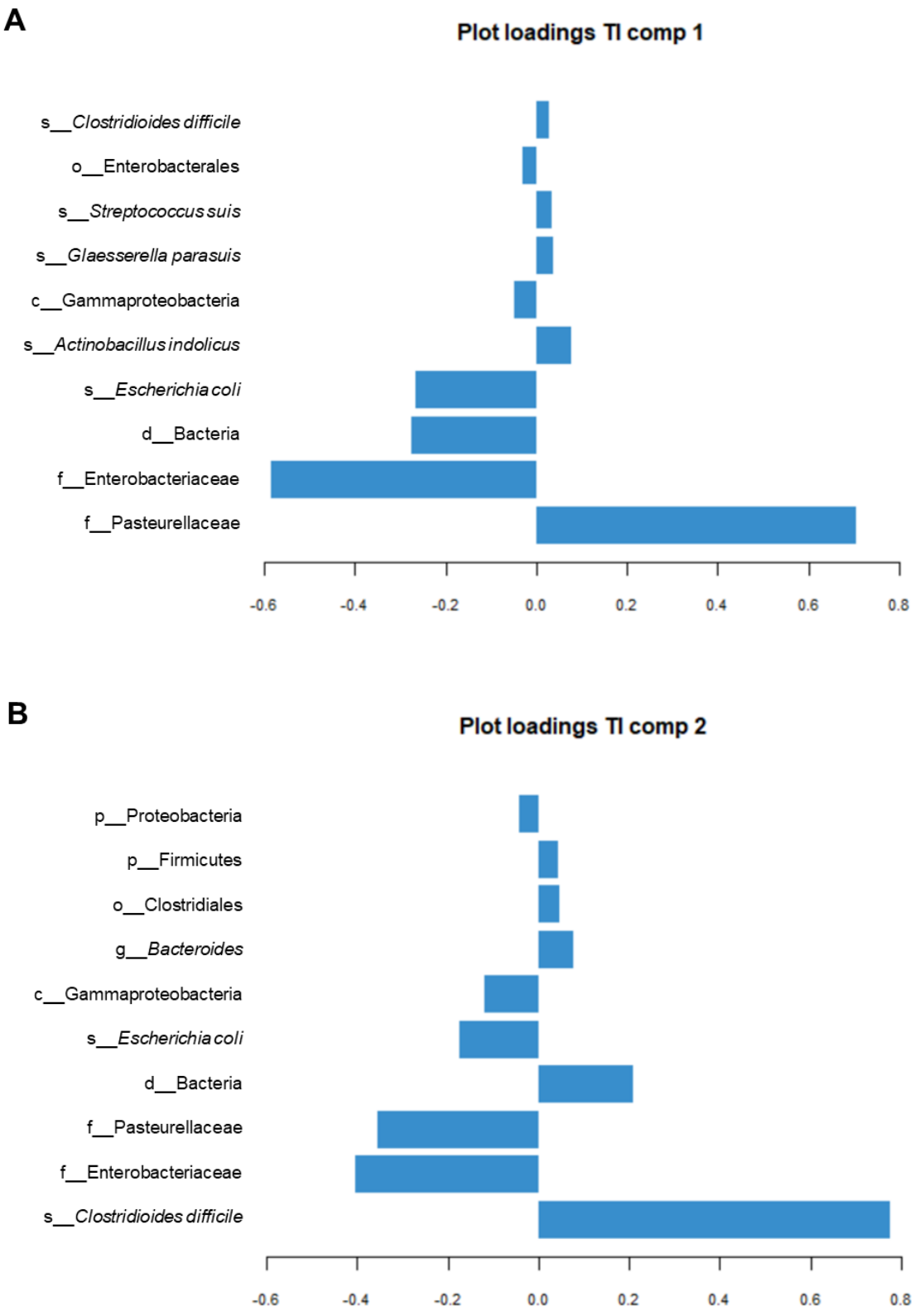

Figure 8. Barplot of top 10 loadings of principal components 1 (A) and 2 (B) derived from the PCA of terminal ileal microbiota of digesta samples as described previously. X-axis shows PCA eigenvalues.

PCoA plots using Unifrac (Figure 9A) and Bray-Curtis (Figure 9B) measures were drawn to examine the dissimilarities between treatment groups. In the Unifrac PCoA plot, pigs fed caprine milk form a distinct cluster (Figure 9A). Some overlap exists for the samples belonging to pigs fed bovine or ovine milk (Figure 9A). A relatively high percentage (77.8%) shows that this clustering covers a large proportion of all variables. However, due to the small sample size, in particular for the caprine group, it is unclear whether these clustering effects are true (Figure 9A). Distinct cluster groupings of samples belonging to pigs fed caprine or ovine milk were observed in Bray-Curtis PCoA (Figure 9B). Apart from one outlier (bottom left of the plot), a cluster group may also be applied to the samples belonging to pigs fed bovine milk. These results suggest that the three treatment groups were distinct from one another. Again, a high percentage encompassed by the plot (85.3%) gives confidence that this finding includes a large majority of variables.

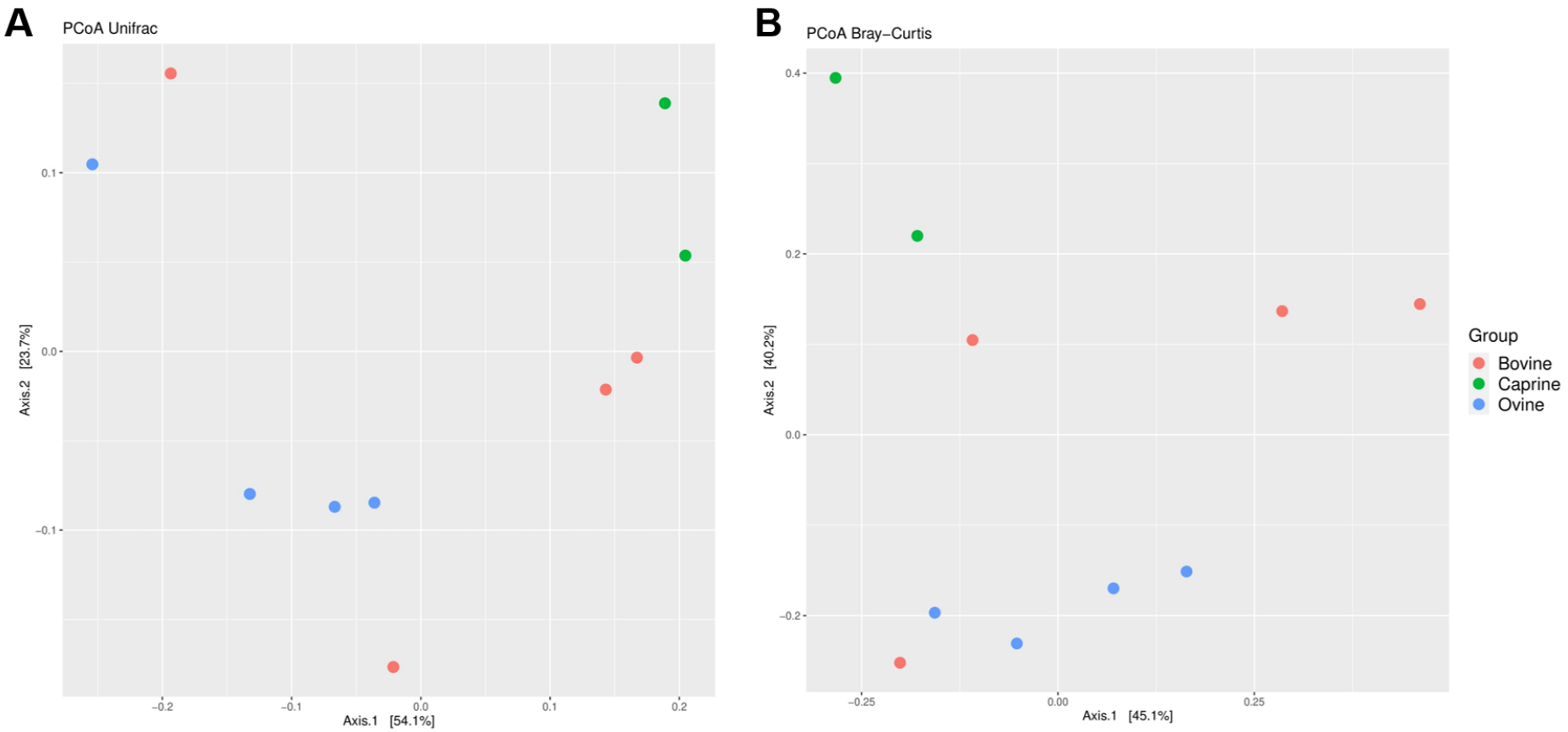

Figure 9. PCoA Unifrac (A) and Bray-Curtis (B) plots of terminal ileal microbiota of digesta samples as described previously. Key: Red – bovine; green – caprine; blue – ovine.

Next, a supervised method (sPLS-DA) was used to allow for further discernment of any cluster groups (Figure 10). Again, samples belonging to pigs fed caprine milk form a distinct cluster, with overlaps evident for pigs fed bovine or ovine milk (Figure 10). Clusters for ovine and caprine groups were more compact, whereas the bovine cluster was more spread out (Figure 10).

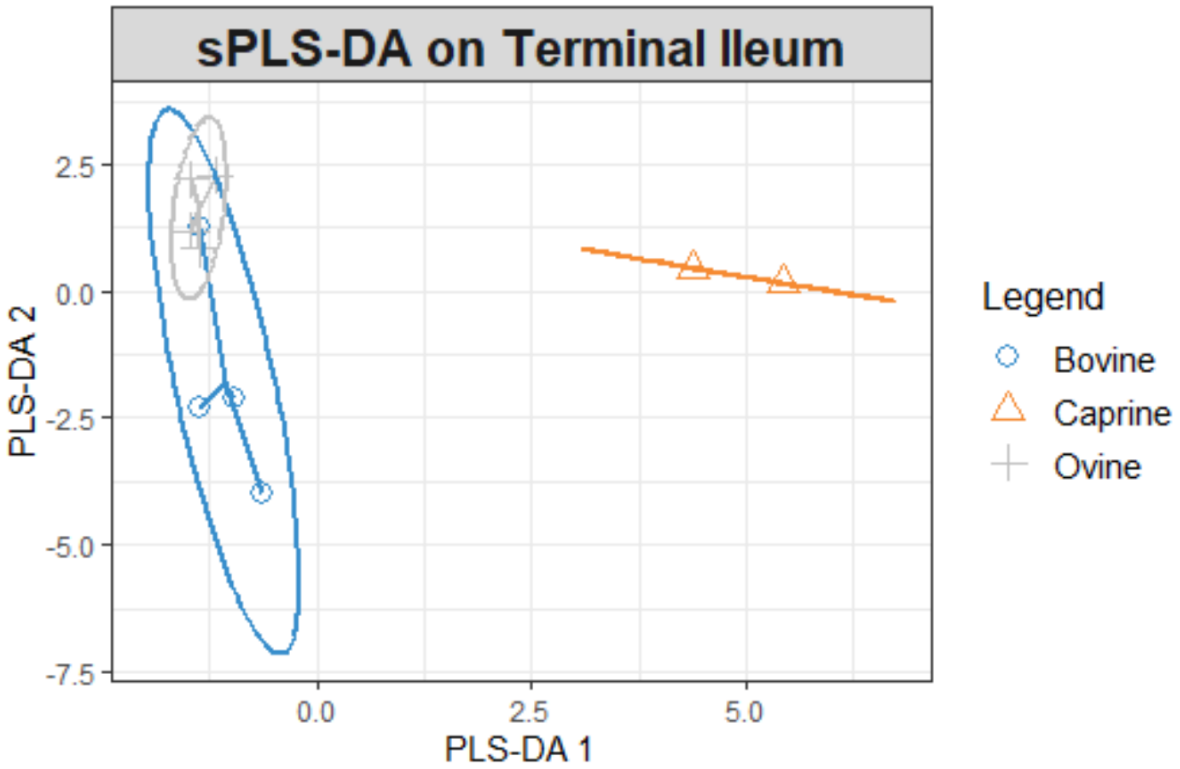

Figure 10. sPLS-DA plot of terminal ileal microbiota of digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. Confidence ellipses (80%) and centroid for each treatment group are also drawn.

Plot loadings of components 1 (Figure 11A) and 2 (Figure 11B) were visualised in barplots. For component 1, bacteria belonging to genus *Subdoligranulum* and *Desulfovovibrio piger* found in pigs fed caprine milk had the greatest influence on this variable (Figure 11A). Conversely, for component 2, unclassified bacteria belonging to orders Fusobacteriales and Bacillales from pigs fed bovine milk had the greatest (inverse) influence on this variable (Figure 11B).

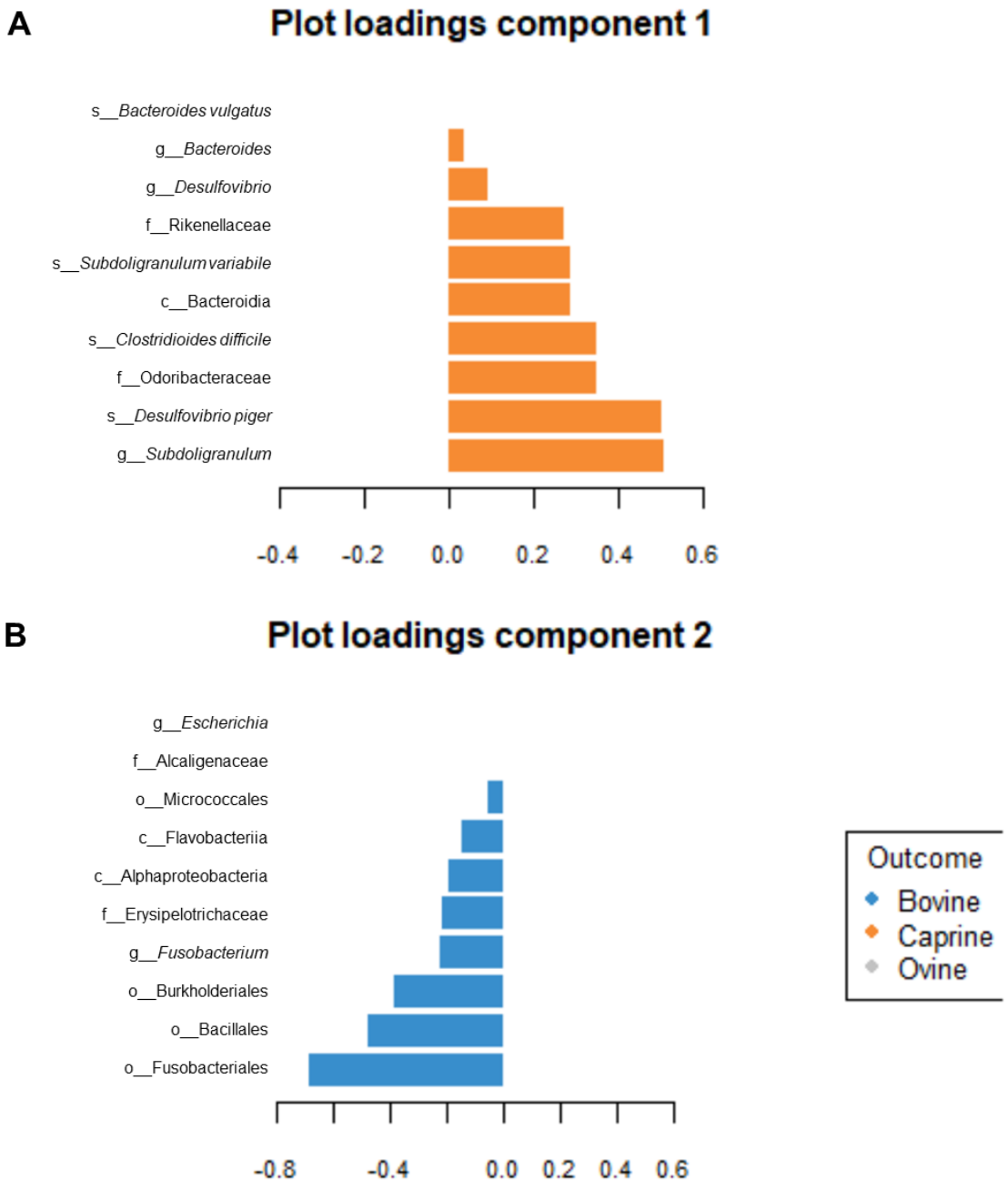

Figure 11. Barplot of top 10 loadings of component 1 (A) and 2 (B) derived from the sPLS-DA of terminal ileal microbiota of digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. X-axis shows eigenvalues.

The total abundance (standardised) of each sample at the phylum level was visualised in a stacked bar-plot (Figure 12A). The relative abundance is shown in Figure 12B. Family and genus levels' relative abundances are shown in Figures 13A and 13B. ANCOMBC analyses of terminal ileal microbial reads at genus and family levels identified multiple differentially abundant taxa between milk treatments

(Figure 13). In pigs fed bovine and ovine milk, the largest proportion of bacteria belonged to phylum Proteobacteria, whereas it was phylum Firmicutes in pigs fed caprine milk (Figure 12). In pigs fed bovine milk, the largest proportion of bacteria belonged to family Pasteurellaceae, however this was not consistent between all samples (Figure 16A). In pigs fed caprine milk, the largest proportion of bacteria belonged to family Peptostreptococcaceae (Figure 13A). In pigs fed ovine milk, the largest proportion of bacteria belonged to family Enterobacteriaceae (Figure 13A). None of these observed differences between treatment groups were statistically significant. At the genus level, the largest proportion of bacteria was unclassified (unlabelled pink top group), which was consistent for all treatment groups (Figure 13B). An increased relative abundance of bacteria belonging to genus *Clostridioides* was also evident, however this was not significant (Figure 13B).

Bacteria belonging to the genus *Desulfovibrio* were not present in pigs fed bovine or ovine milk but present in pigs were caprine milk (p=0, q=0, w=210) (Figure 14A). Further, pigs fed ovine milk did not harbour any bacteria belonging to genera *Blautia* (p=0, q=0, w=135) (Figure 14B), *Ruminococcus* (p=0, q=0, w=122) (Figure 14C) or *Prevotella* (p=0, q=0, w=110) (Figure 14D). Bacteria belonging to genera *Blautia*, *Ruminococcus* or *Prevotella* was present in pigs fed bovine or caprine milk (Figures 14B, 14C, 14D).

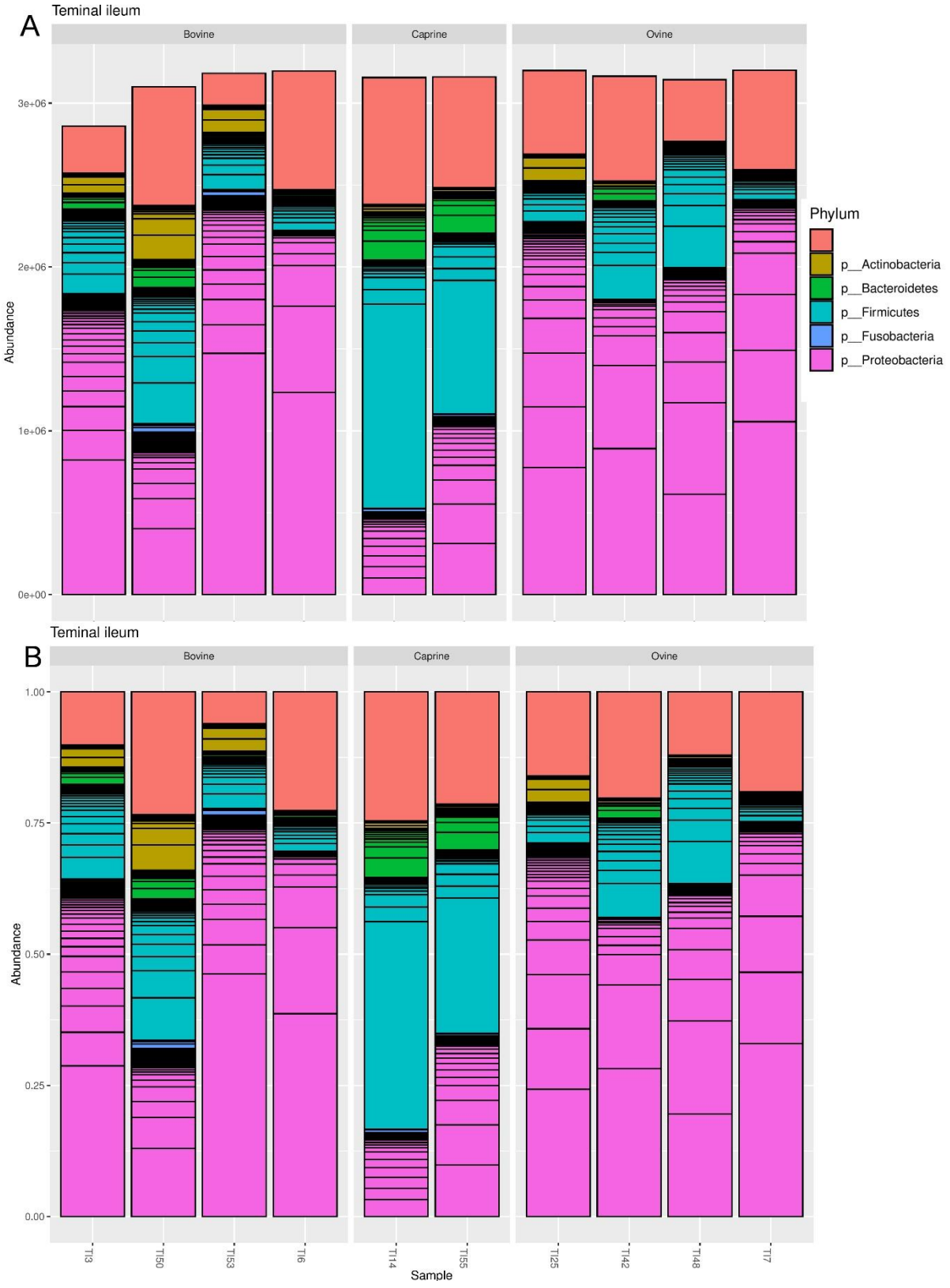

Figure 12. A: Stacked bar-plot after standardisation of the total abundance of terminal ileal microbiota at phylum level of digesta samples as described previously. B: Stacked bar-plot of the same data converted to relative abundance.

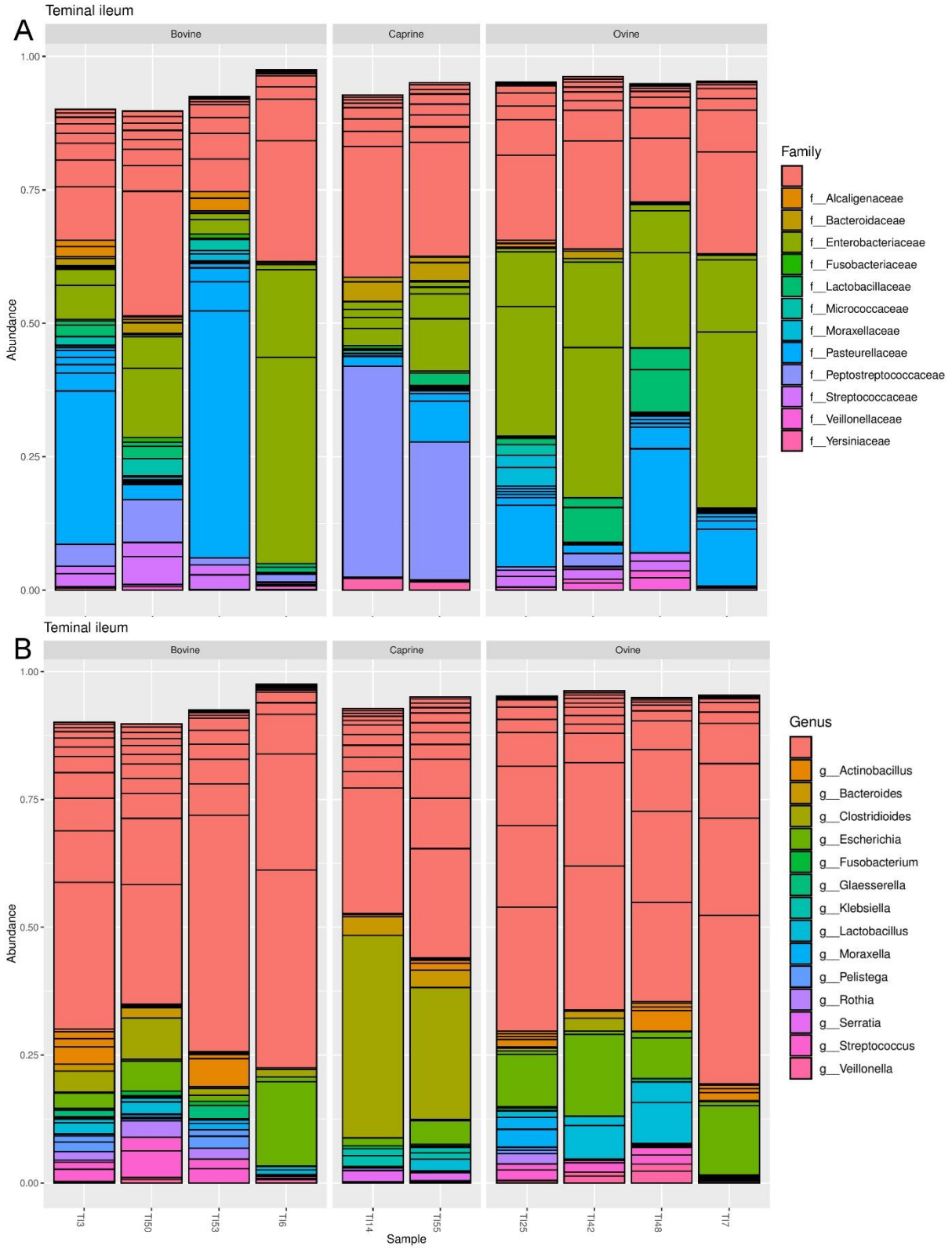

Figure 13. A: Stacked bar-plot after standardisation of the relative abundance of top 20 taxa of terminal ileal microbiota at family level in digesta samples as described previously. B: Stacked bar-plot of the same parameters of the top 20 taxa at the genus level. Both graphs primarily consist of unclassified bacteria.

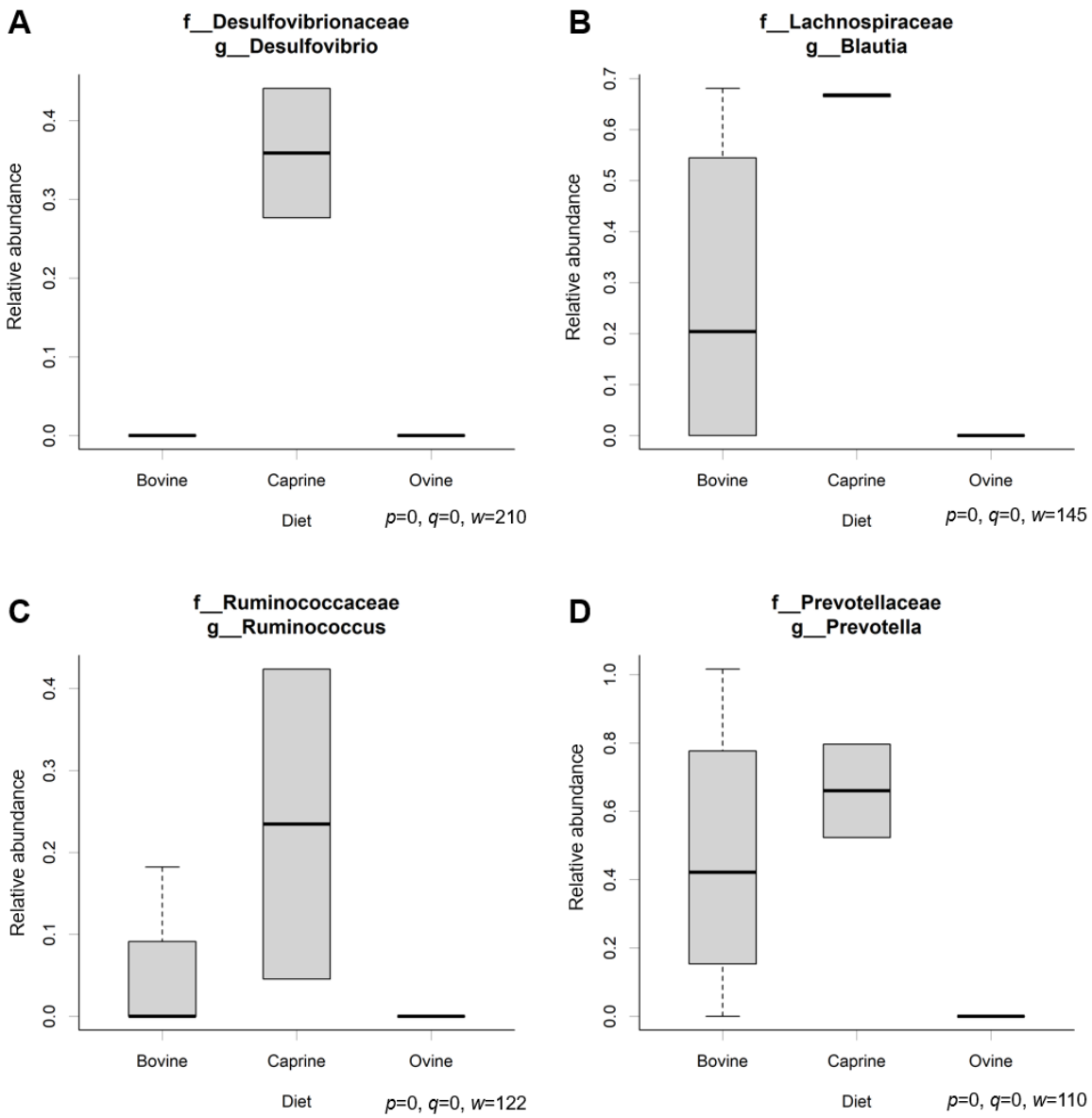

Figure 14. Box plots of the relative abundance of statistically significant taxonomic genera of terminal ileal microbiota of digesta samples collected as described previously. A: *Desulfovibrio*; B: *Blautia*; C: *Ruminococcus*; D: *Prevotella*. Global *p* value, significant when p<0.05. *q* value is the *p* value adjusted for the false discovery rate. *w* value reports the number of times the null hypothesis was rejected.

2.4.4.2. Colonic microbiota

Next, the proximal colon was assessed (n=23). A PCA plot was drawn to examine similarities between treatment groups (Figure 15). Again, no discernible clusters were observed, and the percentage of variables was lower (57%) than in the terminal ileum.

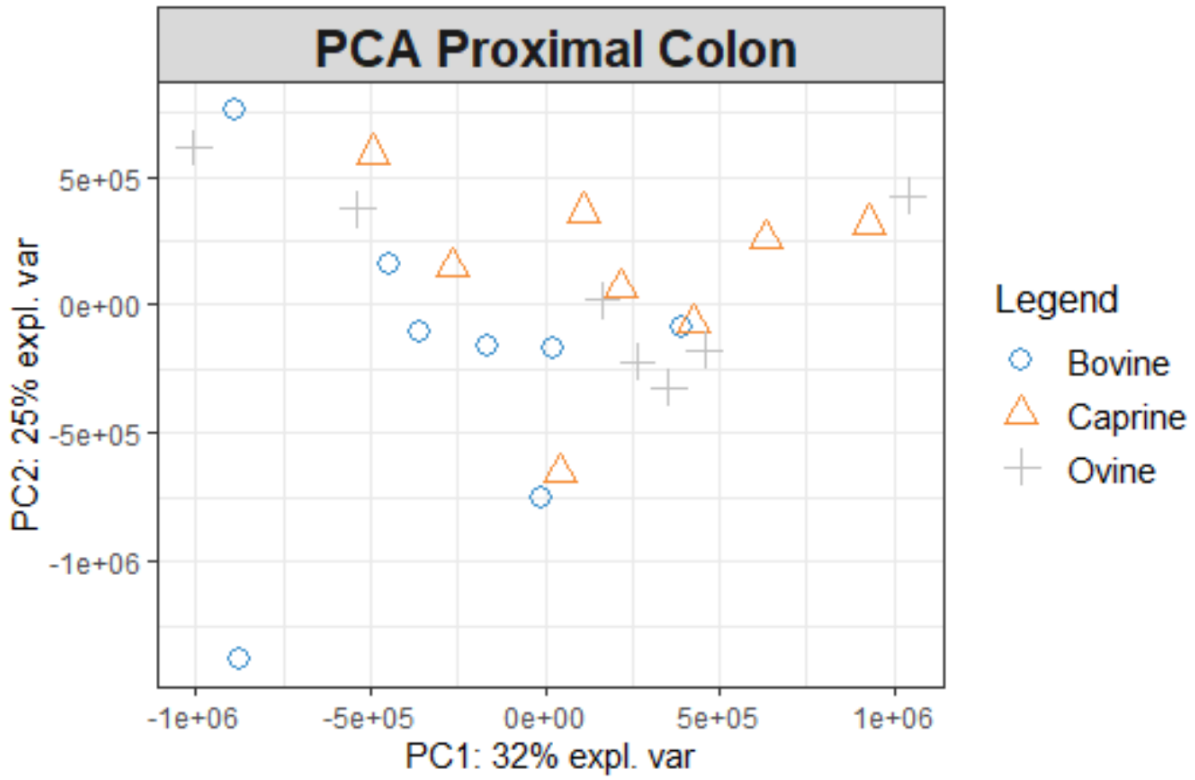

Figure 15. PCA plot of proximal colonic microbiota of digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine.

Plot loadings of the top 10 taxa influencing components 1 (Figure 16A) and 2 (Figure 16B) in the PCA were then visualised in barplots. For principal component 1, bacteria belonging to the genus *Bacteroides* had the greatest influence (Figure 16A). Bacteria belonging to phylum Firmicutes and order Clostridiales had the greatest influence on principal component 2 (Figure 16B). All other taxa had minimal influence on both principal components 1 and 2 (Figures 16A and 16B).

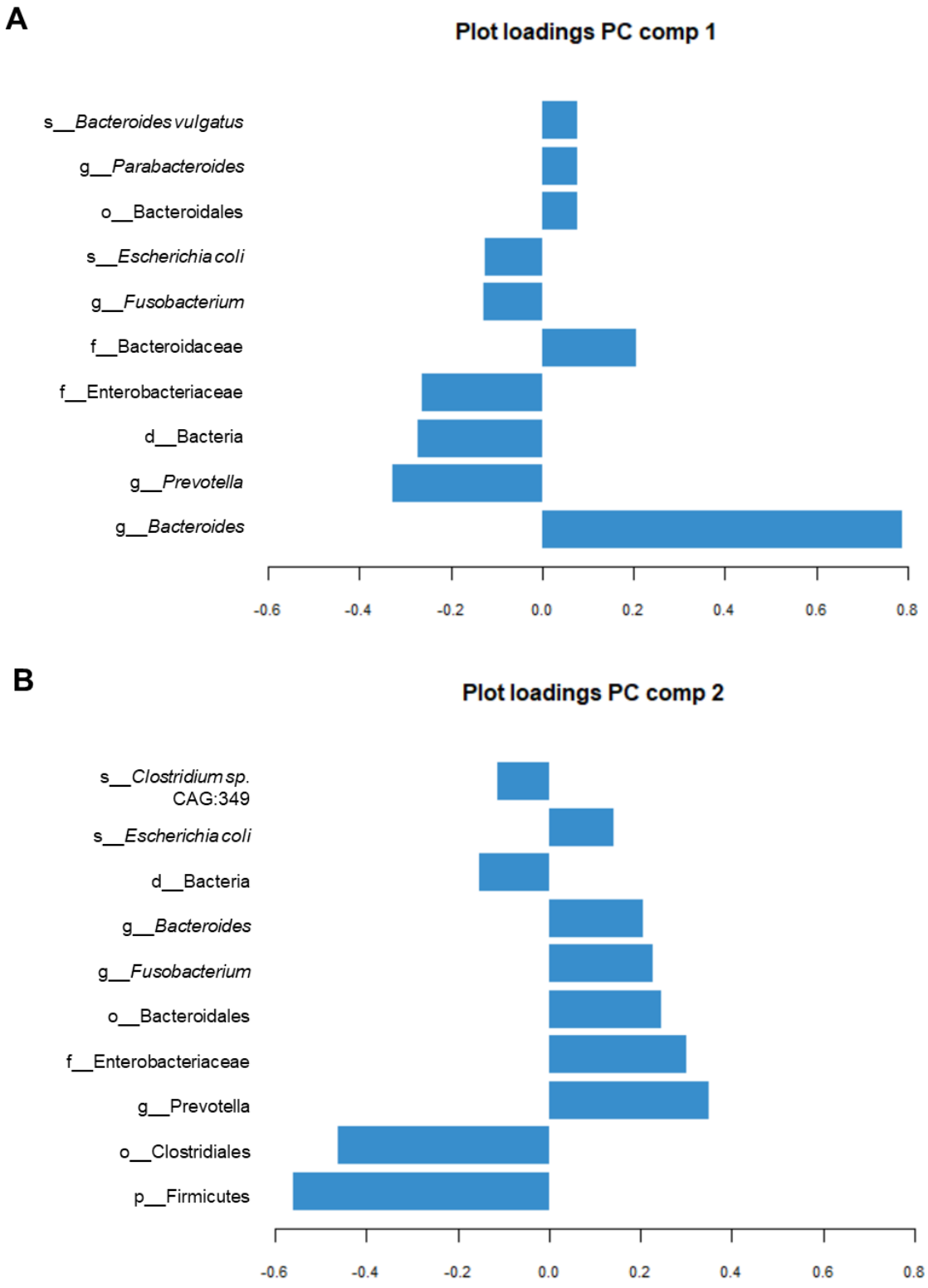

Figure 16. Barplot of top 10 loadings of principal components 1 (A) and 2 (B) derived from the PCA of proximal colonic microbiota of digesta samples as described previously.

PCoA plots using Unifrac (Figure 17A) and Bray-Curtis (Figure 17B) measures were drawn to examine the dissimilarities between treatment groups. No separation of cluster groups can be observed in either plot, suggesting that no milk treatments were distinct from each other. However, as compared with the same graph type drawn for the terminal ileal microbiota (Figure 9), the percentage of variables in the colonic microbiota was lower (46.5% and 47.6% for Unifrac and Bray-Curtis, respectively). This result suggests that variability within the colonic dataset was much greater.

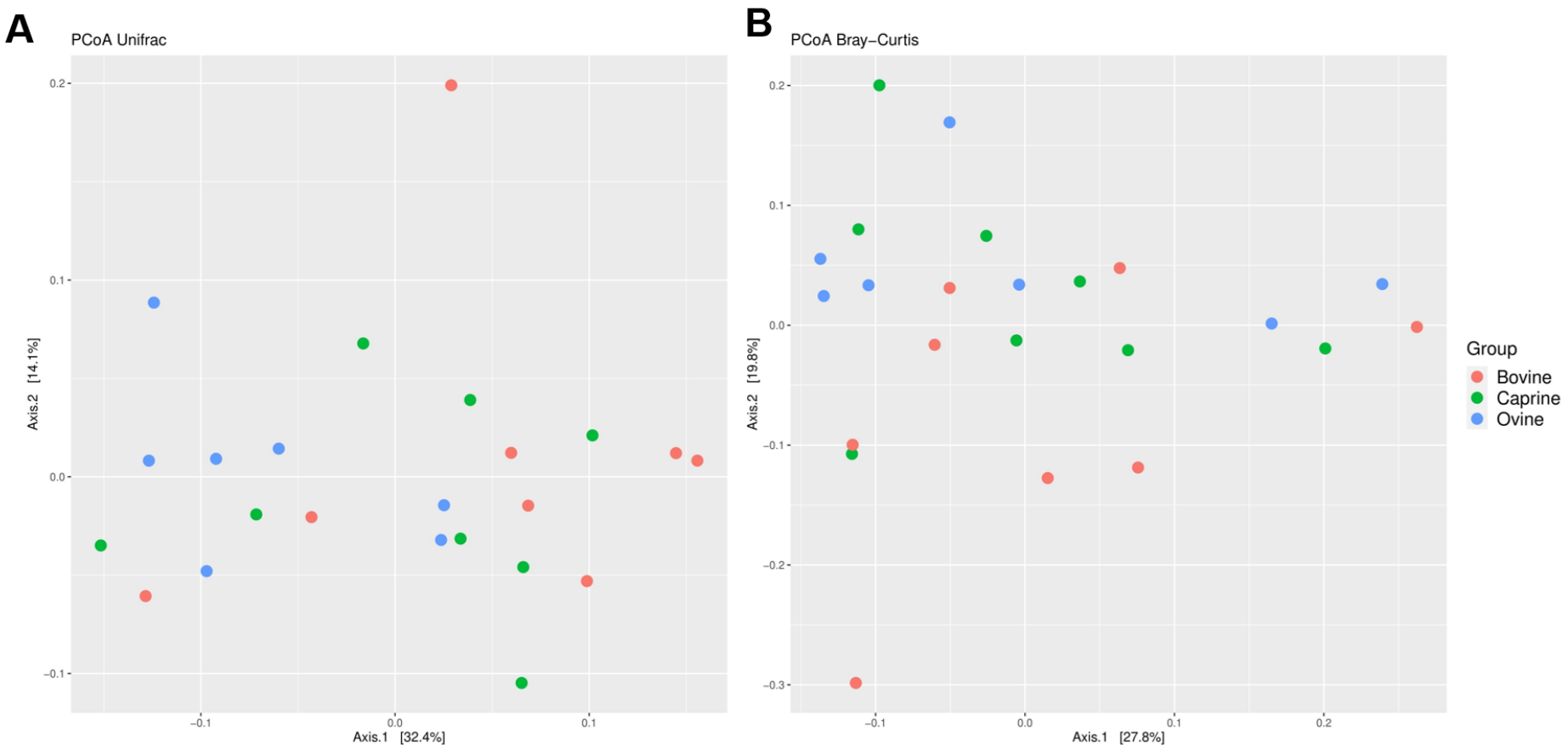

Figure 17. PCoA Unifrac (A) and Bray-Curtis (B) plots of proximal colonic microbiota of digesta samples as described previously. Key: Red – bovine; green – caprine; blue – ovine.

Next, a supervised method (sPLS-DA) was used to allow for further discernment of any cluster groups (Figure 18). More distinct clustering was observed for the bovine and caprine groups, with samples belonging to the ovine group quite spread out (Figure 18).

Plot loadings of components 1 (Figure 19A) and 2 (Figure 19B) were similarly visualised in barplots. For component 1, bacteria belonging to order Victivalalles, *Victivallis vadensis* and phylum Lentisphaerae (also known as Lentisphaerota) found in pigs fed ovine milk had the greatest influence (Figure 19A). For component 2, bacteria belonging to the genus *Bacteroides* and family *Bacteroidacaea* found in pigs fed caprine milk had the greatest influence. Further, bacteria belonging to order Clostridiales found in pigs fed bovine milk also had some influence on component 2 (Figure 19B). All other taxa had minimal influence on both components 1 and 2 (Figures 19A, 19B).

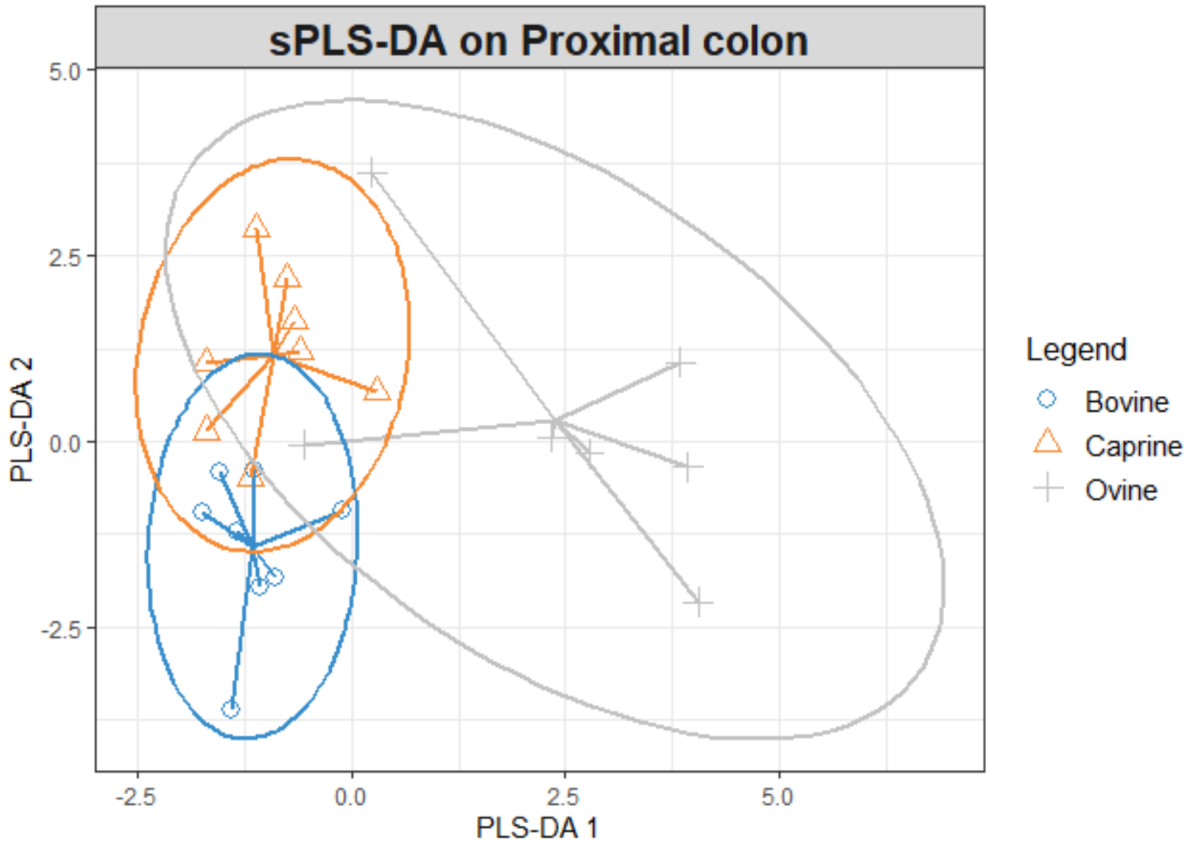

Figure 18. sPLS-DA plot of proximal colonic microbiota of digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. Confidence ellipses (80%) and centroid for each treatment group are also drawn.

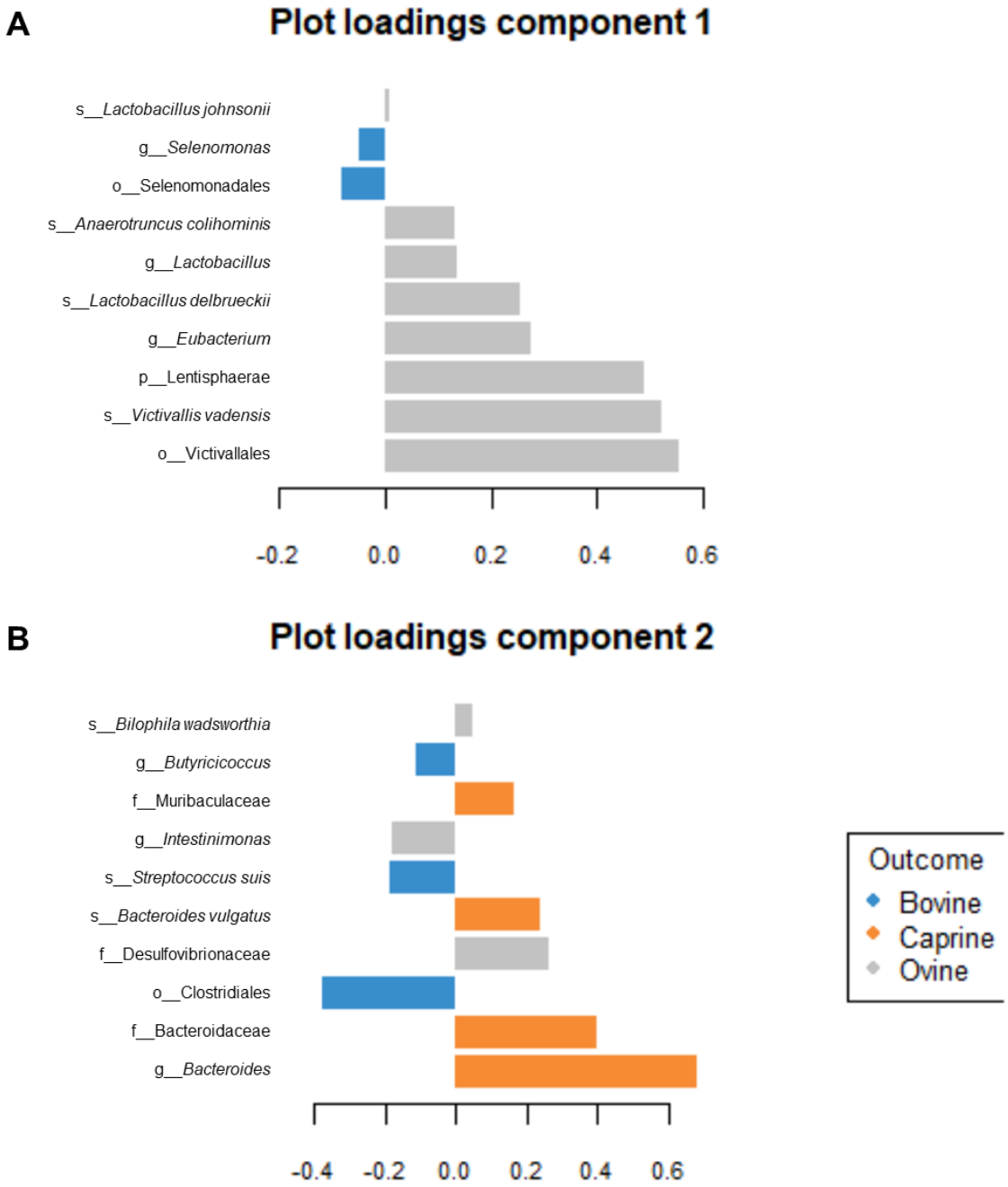

Figure 19. Barplot of top 10 loadings of component 1 (A) and 2 (B) derived from the sPLS-DA of proximal colonic microbiota of digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. X-axis shows eigenvalues.

The total abundance (standardised) of each proximal colon sample at the phylum level was visualised in a stacked bar-plot (Figure 20A). The relative abundance as a proportion is shown in Figure 20B. The largest proportion of bacteria present across all three treatment groups belonged to phyla Bacteroidetes and Proteobacteria (Figure 20). The relative abundances at family and genus levels are

shown in Figures 21A and 21B. A large portion of unclassified bacteria at both family and genus levels was evident in Figure 21 (unlabelled pink top group), which has been observed in other piglet microbiota data.[227-229]

ANCOMBC statistical analysis found significant differences between milk treatments in three genera (Figure 22). When compared with pigs fed bovine or caprine milk, pigs fed ovine milk did not harbour any bacteria belonging to genera *Selenomonas* (p=0, q=0, w=17) (Figure 22A) or *Elusimicrobium* (p=0, q=0, w=13) (Figure 22B). In addition, bacteria belonging to the genus *Salmonella* were not present in pigs fed bovine or caprine milk but they were present in pigs fed ovine milk (p=0, q=0, w=4) (Figure 22C). Bacteria belonging to genera *Selenomonas* or *Elusimicrobium* was present in pigs fed bovine or caprine milk at approximate levels to one another (Figures 22A, 22B).

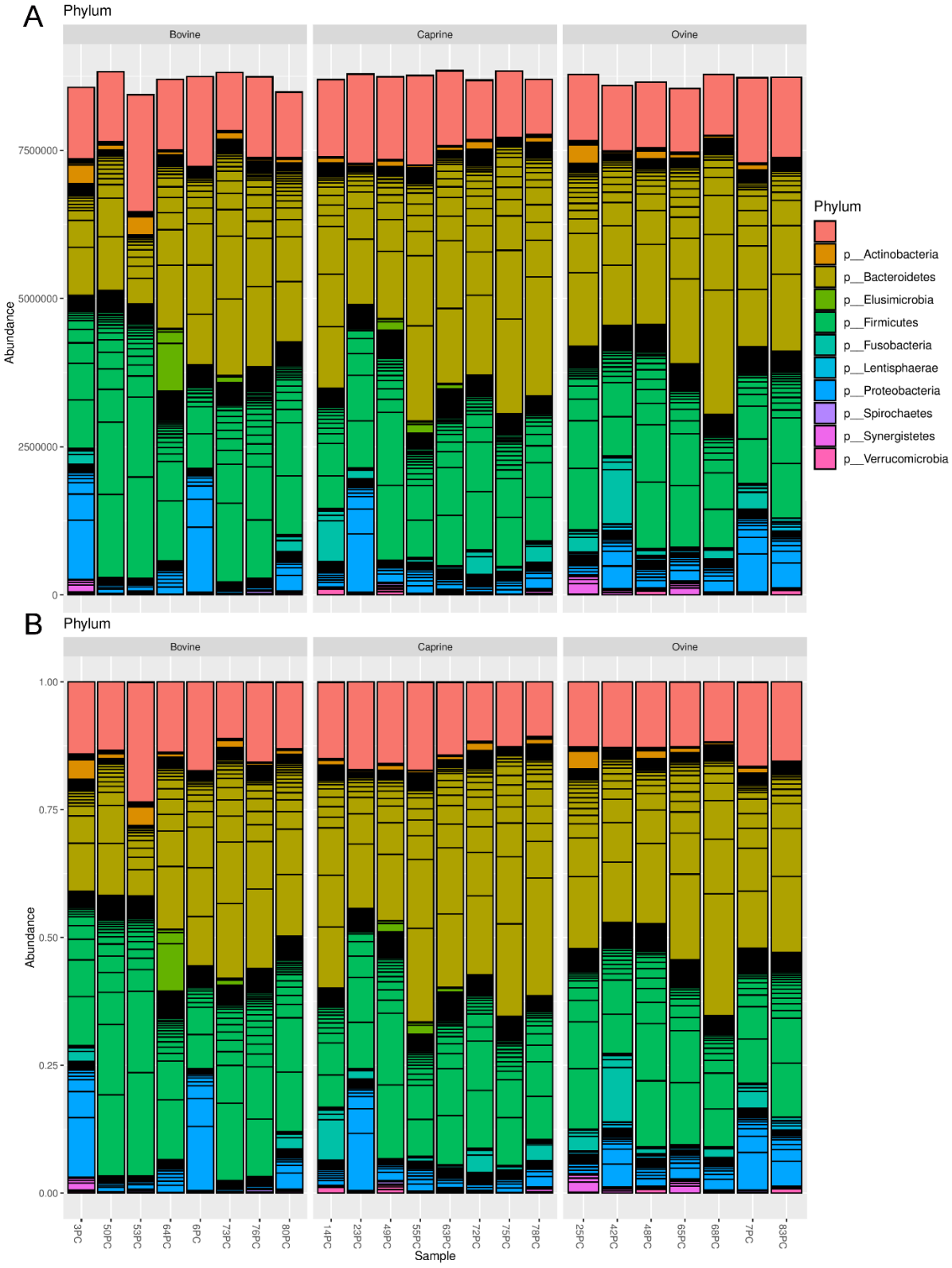

Figure 20. A: Stacked bar-plot after standardisation of the total abundance of proximal colonic microbiota at the phylum level in digesta samples as described previously. B: Stacked bar-plot of the same data converted to relative abundance.

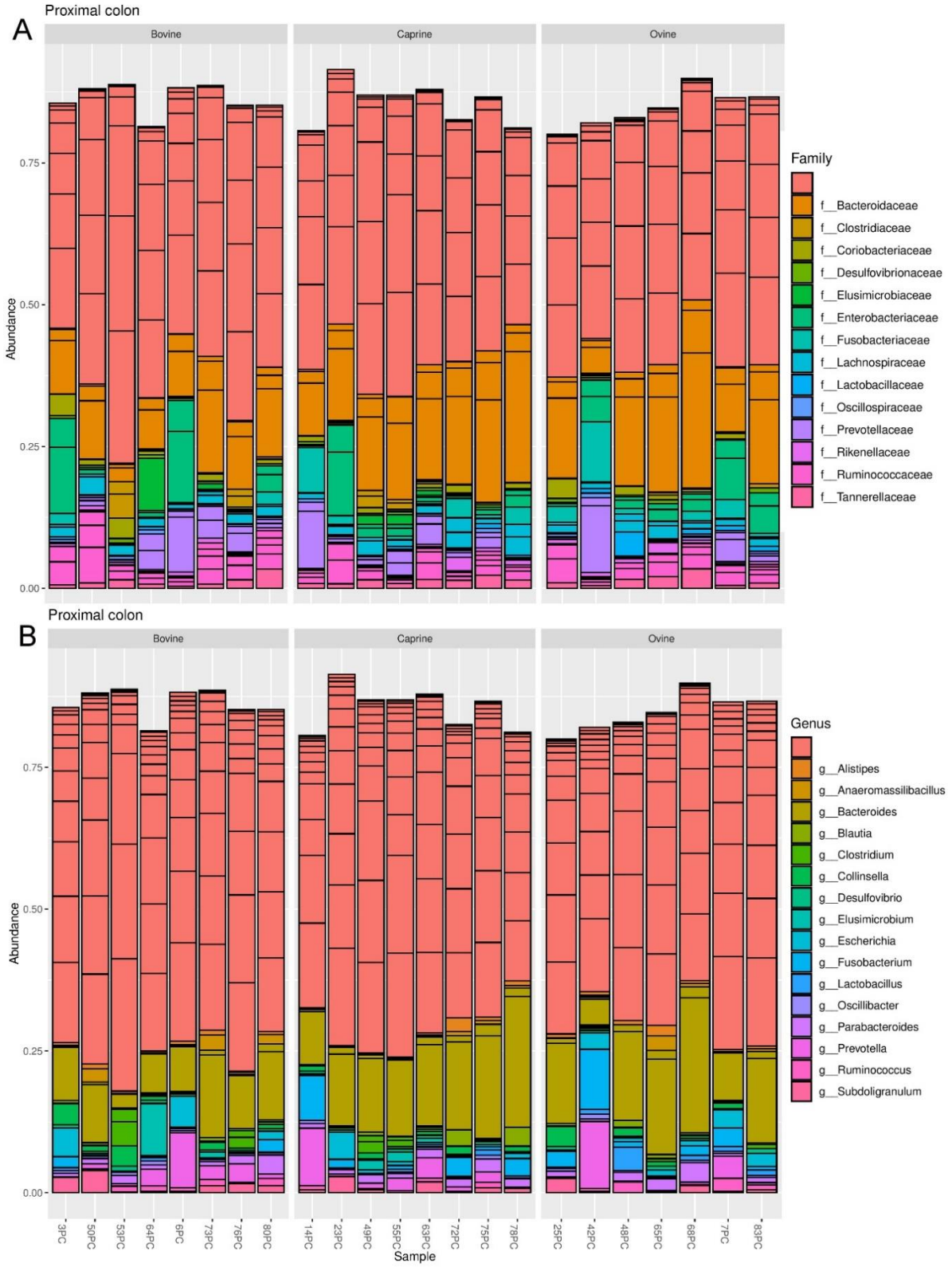

Figure 21. A: Stacked bar-plot of top 20 taxa at family level after standardisation of the relative abundance of the proximal colonic microbiota in digesta samples as described previously. B: Stacked bar-plot of the same parameters of the top 20 taxa at the genus level.

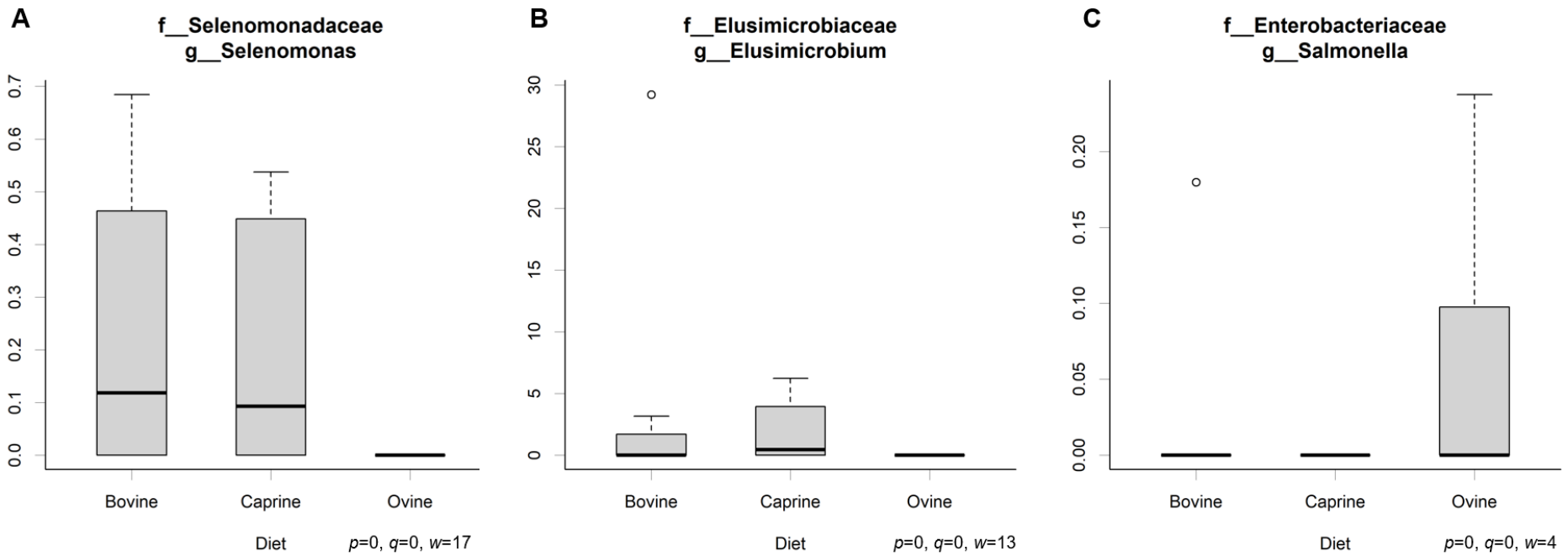

Figure 22. Box plots of the relative abundance of statistically significant taxonomic genera of proximal colonic microbiota of digesta samples. A: *Selenomonas*; B: *Elusimicrobium*; C: *Salmonella*. Global p value, significant when p<0.05. q value is the p value adjusted for the false discovery rate. w value reports the number of times the null hypothesis was rejected.

2.4.5. Function

2.4.5.1. Terminal ileum

PCoA plots using Unifrac (Figure 23A and C) and Bray-Curtis (Figure 23B and D) measures were drawn to examine the dissimilarities between treatment groups. The caprine group was distinctly separate from the other treatment groups in both KEGG and SEED Unifrac measures (Figures 23A and C) and the KEGG Bray-Curtis measure (Figure 23B) but not in the SEED Bray-Curtis measure (Figure 23D). Bovine and ovine group clusters overlap in all instances (Figures 23A, 23B).

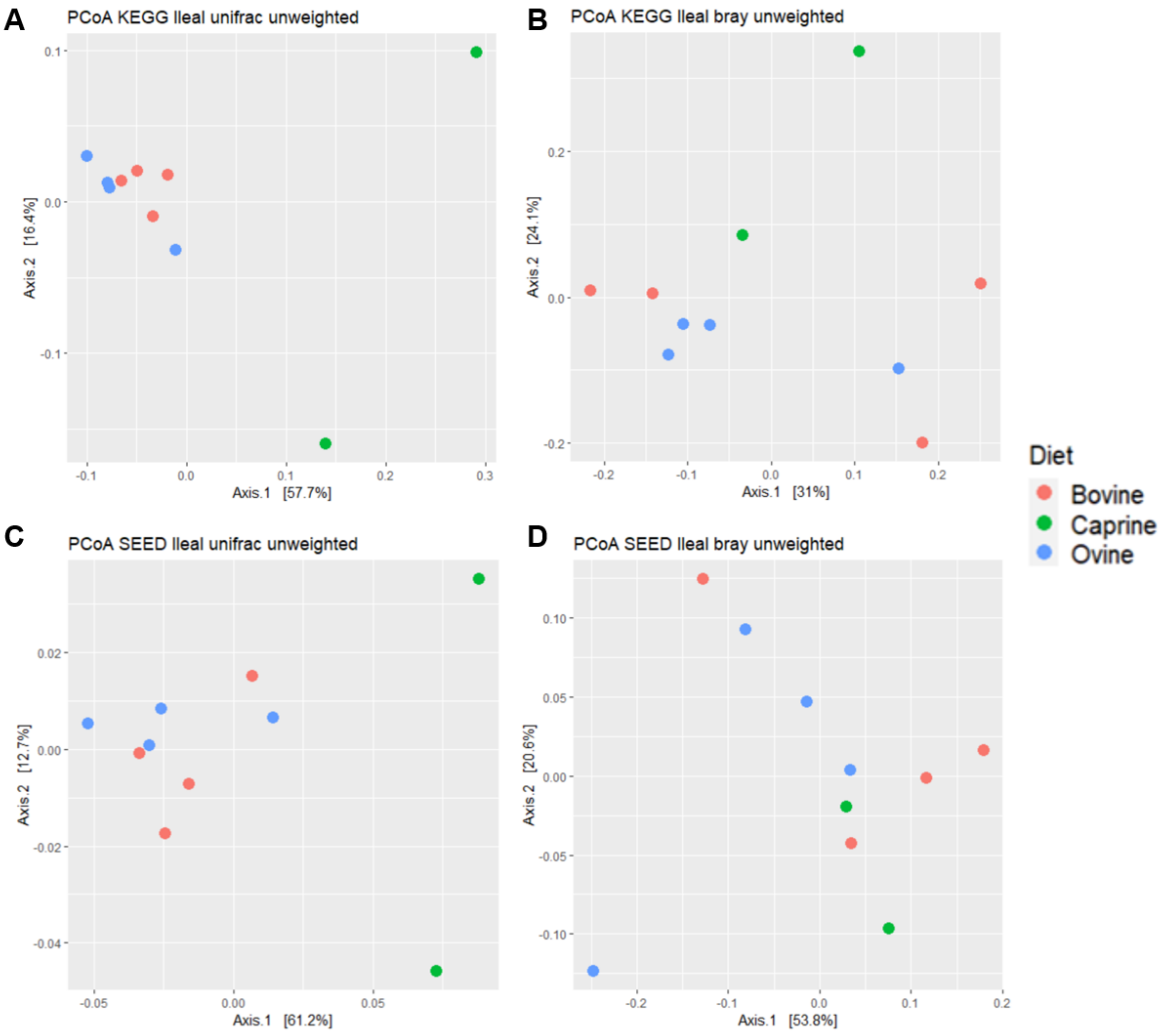

Figure 23. PCoA Unifrac (A) and Bray-Curtis (B) plots of terminal ileal KEGG metagenome in digesta samples as described previously. PCoA Unifrac (C) and Bray-Curtis (D) plots of terminal ileal SEED metagenome in digesta samples as described previously. Key: Red – bovine; green – caprine; blue – ovine.

Next, a supervised method (sPLS-DA) was used to allow for further discernment of any cluster groups, firstly for the KEGG metagenome (Figure 24) and then for the SEED metagenome (Figure 26). For KEGG, all three treatment groups formed distinct clusters, but the caprine group remains the furthest removed, suggesting that the bovine and ovine groups were more similar to each other than the caprine group (Figure 24).

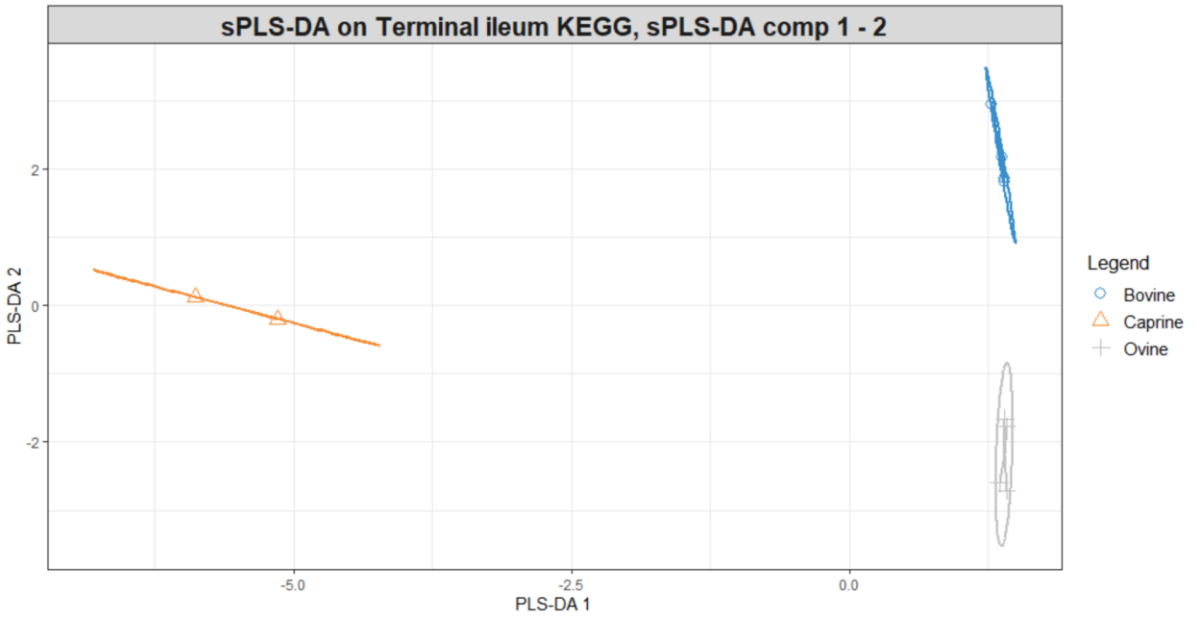

Figure 24. sPLS-DA plot of terminal ileal KEGG metagenome in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. Confidence ellipses (80%) and centroid for each treatment group are also drawn.

Plot loadings of components 1 (Figure 25A) and 2 (Figure 25B) were then visualised in barplots. For plot loading component 1, all top ten KEGG classes belonged to the caprine group (Figure 25A). This result is aligned with what was observed in Figure 24, as the caprine group was the furthest removed from the other treatment groups, and component 1 is displayed on the x-axis. The top two KEGG classes that had the most effect on component 1 were 'U3 small nucleolar RNA-associated protein 12' and 'coronin-1B/1C/6', both belonging to the caprine group (Figure 25A). For loading component 2, the KEGG class with the most influence also belonged to the caprine group, namely 'tyrosine-protein kinase Etk/Wzc' (Figure 25B). The other nine KEGG classes belonged to the bovine and ovine groups, which separated them along the y-axis, as shown in Figure 24.

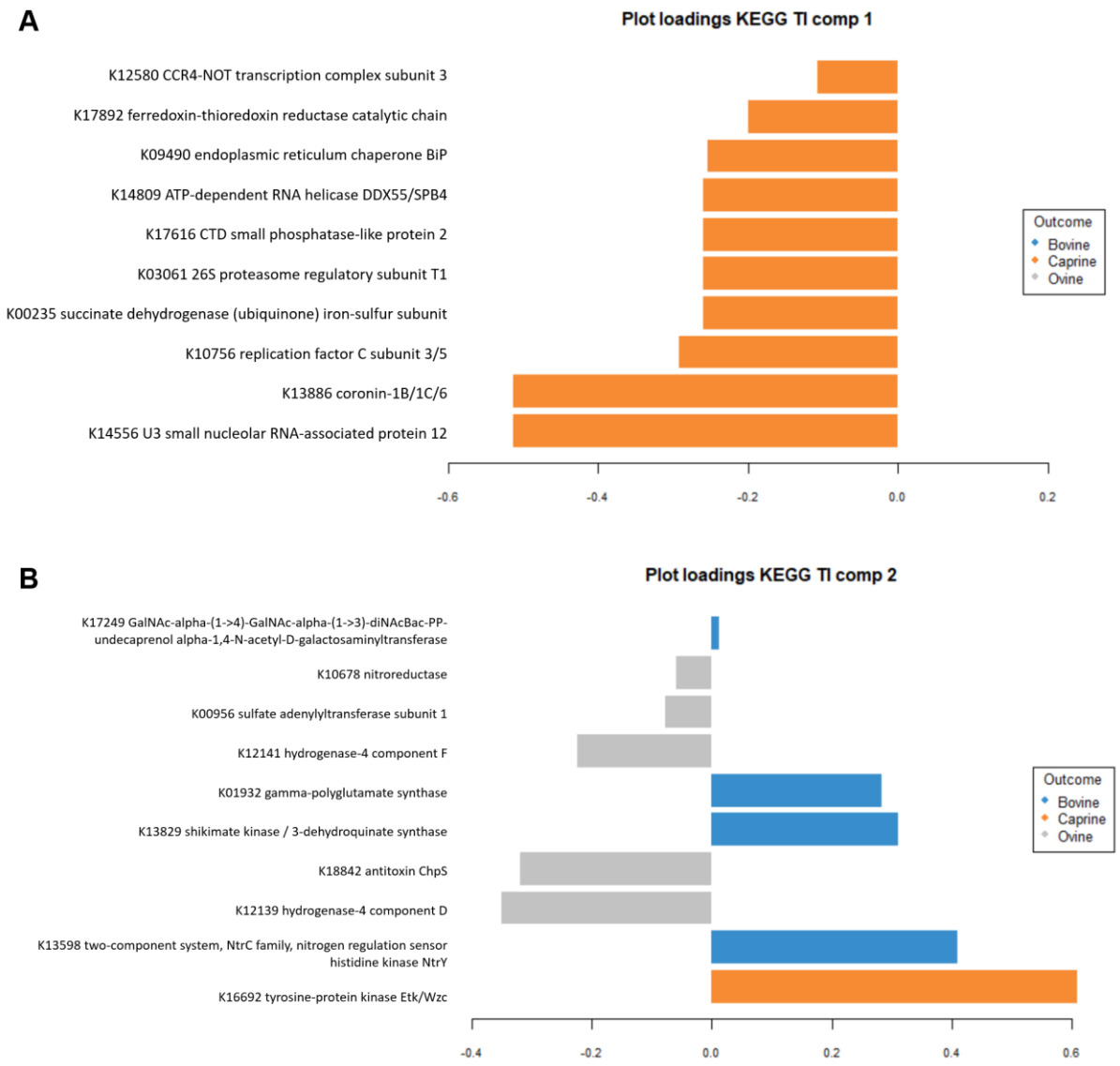

Figure 25. Barplot of top 10 loadings of component 1 (A) and 2 (B) derived from the sPLS-DA plot of terminal ileal metagenome at the lowest KEGG class in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. X-axis shows eigenvalues.

For SEED, the caprine group was the furthest removed from the other two treatment groups (Figure 26). There was some overlap between the bovine and ovine clusters, which suggests they are more similar to each other.

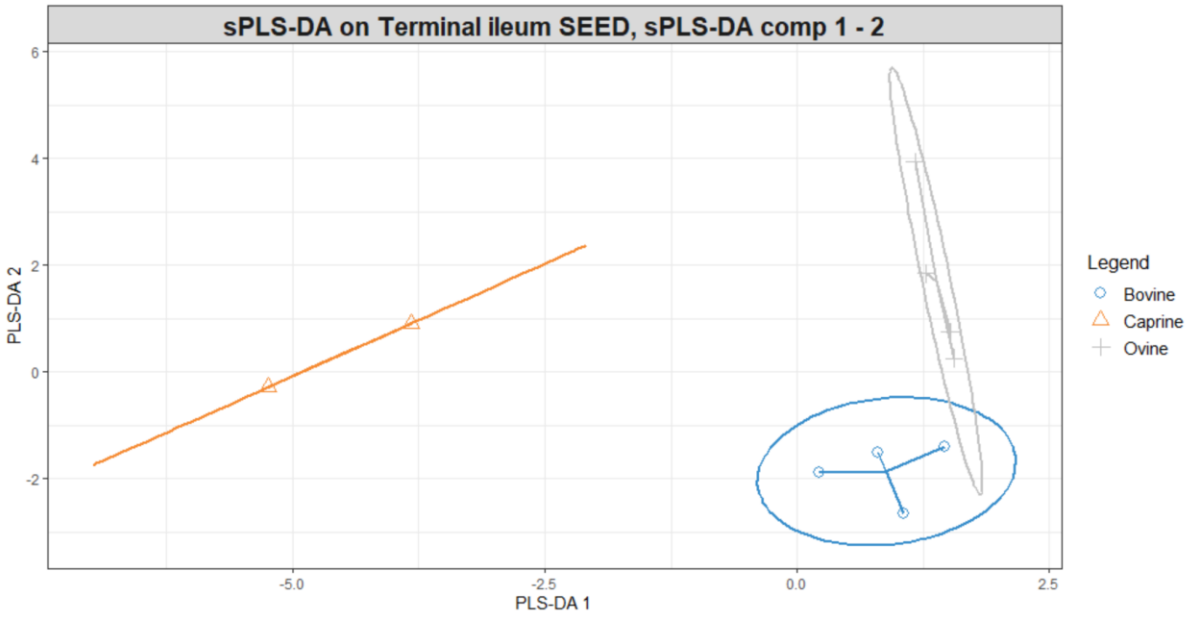

Figure 26. sPLS-DA plot of terminal ileal SEED metagenome in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. Confidence ellipses (80%) and centroid for each treatment group are also drawn.

Plot loadings of components 1 (Figure 27A) and 2 (Figure 27B) were then visualised in barplots. Again, all SEED classes in loading component 1 belonged to the caprine group (Figure 27A), and all SEED classes in loading component 2 belonged to the other two treatment groups (Figure 27B). The SEED class which had the most effect on component 1 was 'sporulation proteins SigEG cluster', belonging to the caprine group (Figure 27A). The SEED class which had the most effect on component 2 was 'YdcE-YdcD toxin-antitoxin systems', belonging to the ovine group (Figure 27B).

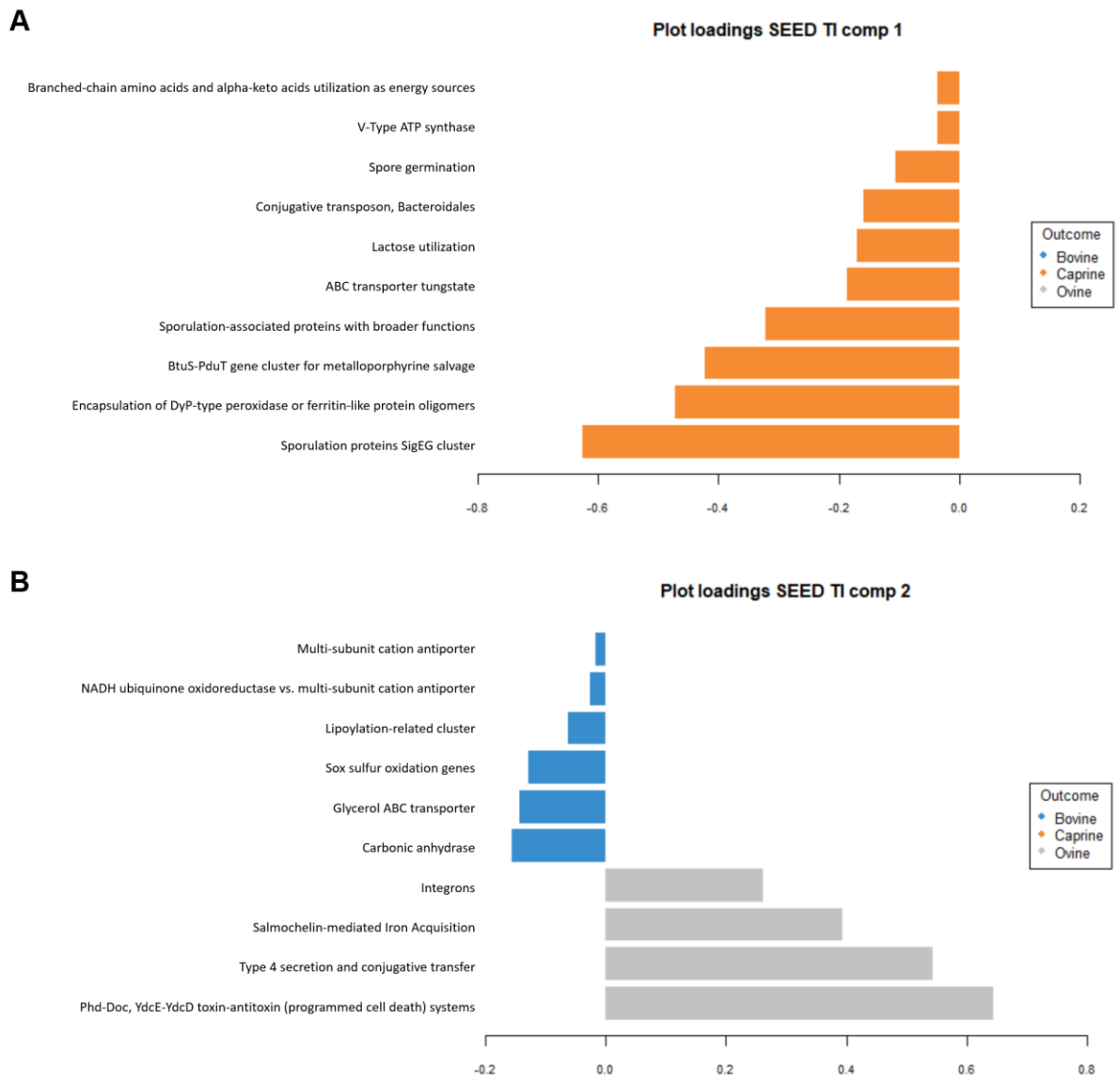

Figure 27. Barplot of top 10 loadings of components 1 (A) and 2 (B) derived from the sPLS-DA plot of terminal ileal metagenome at the lowest SEED class in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. X-axis shows eigenvalues.

Next, the relative abundances (total counts) of genes in the metagenome were linked to KEGG and SEED orthology classes to identify changes in functional potential caused by milk treatments. For KEGG, 'Cellular processes,' 'Organismal systems', and 'Human diseases' functions were removed prior to analysis as they relate to host function rather than microbial, which is not within the scope of this thesis. The gene abundances of the terminal ileal samples at the highest KEGG (Figure 28A) and SEED (Figure 28B) orthology classes (Level 1) were plotted in a stacked bar-plot. The most abundant function

of the ileal metagenome in digesta samples was related to metabolism, regardless of milk treatments (Figure 28). There were no significant differences in KEGG or SEED classes due to treatment.

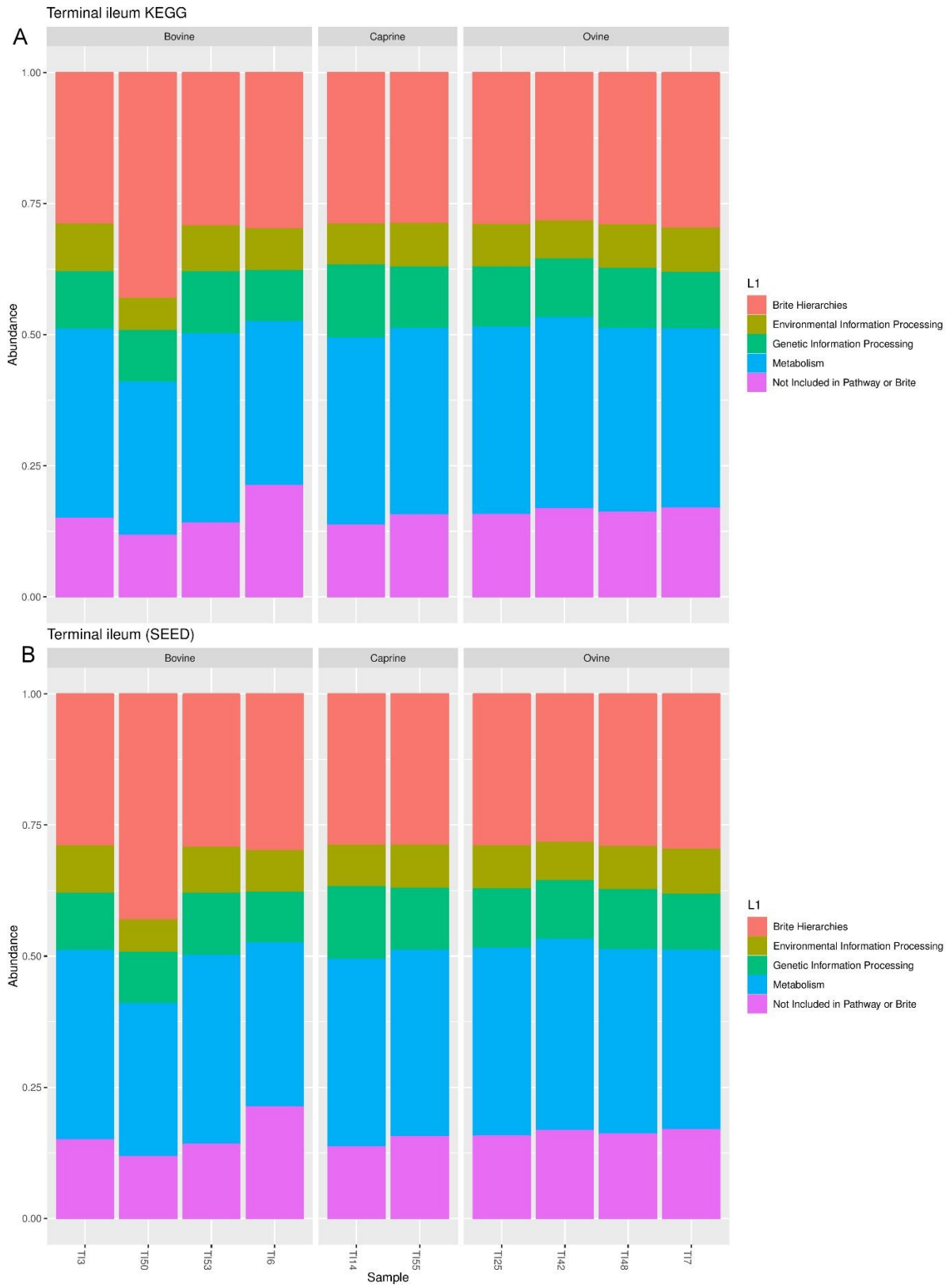

Figure 28. Functional profiles of the terminal ileal metagenome at the highest KEGG (A) and SEED (B) orthology classes (Level 1, L1) in digesta samples as described previously. Colour represents a specific orthology class.

2.4.5.2. Proximal colon

PCoA plots using Unifrac (Figure 29A and C) and Bray-Curtis (Figure 29B and D) measures were drawn to examine the dissimilarities between treatment groups. All treatment groups overlapped with one another in all plots, which suggests that there are no dissimilarities (Figure 29).

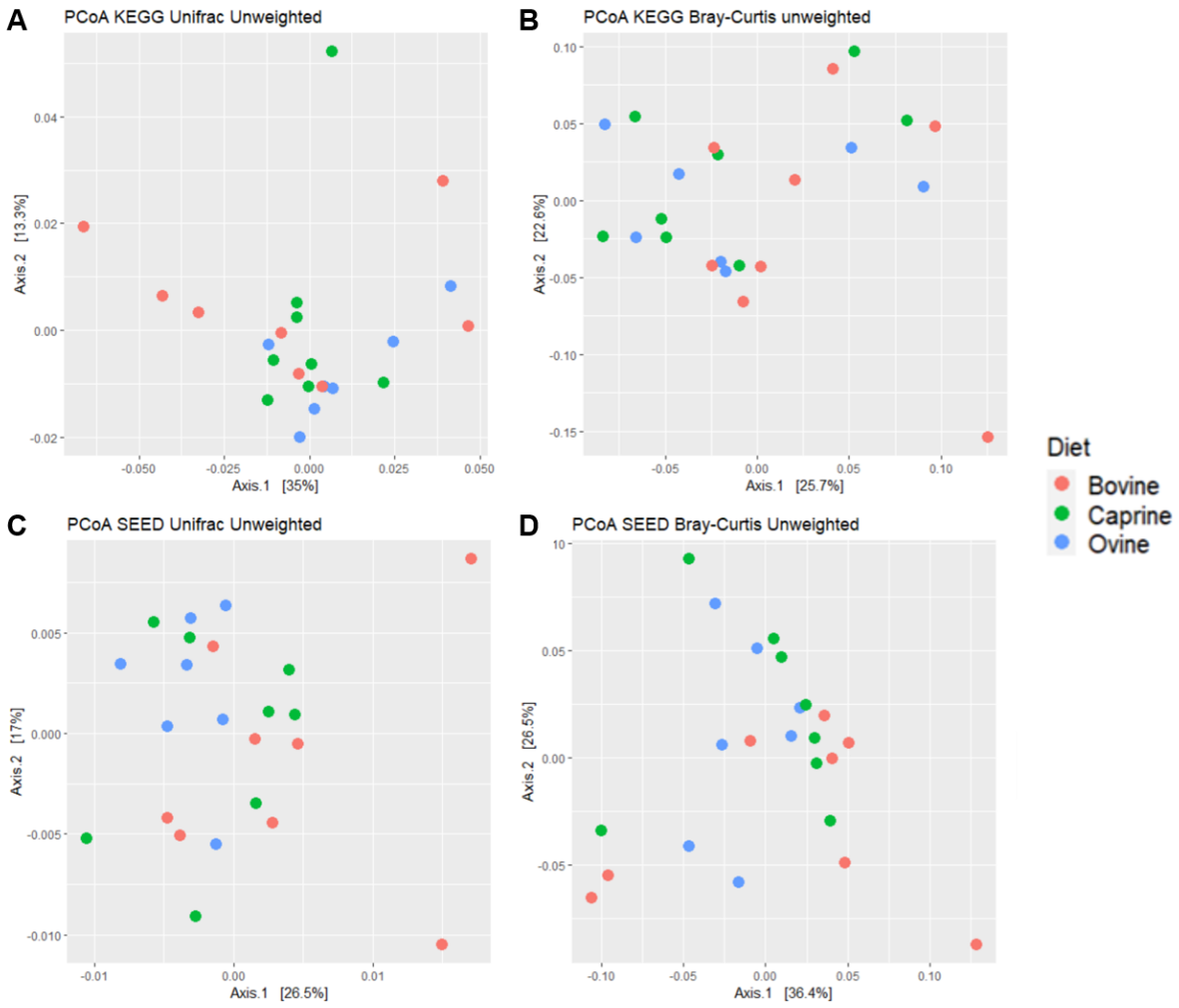

Figure 29. PCoA Unifrac (A) and Bray-Curtis (B) plots of colonic KEGG metagenome in digesta samples as described previously. PCoA Unifrac (C) and Bray-Curtis (D) plots of proximal colonic SEED metagenome in digesta samples as described previously. Key: Red – bovine; green – caprine; blue – ovine.

Next, a supervised method (sPLS-DA) was used to allow for further discernment of any cluster groups, firstly for the KEGG metagenome (Figure 30) and then for the SEED metagenome (Figure 32). For KEGG, whilst there was more separation between each treatment group, some overlap still exists in the confidence ellipses (Figure 30).

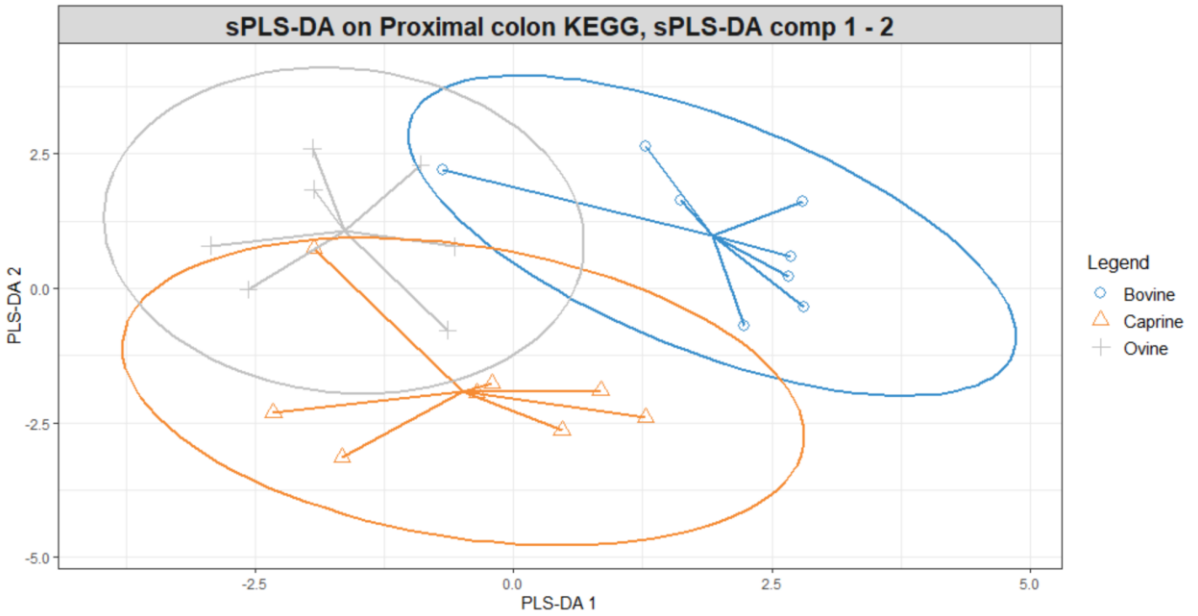

Figure 30. sPLS-DA plot of proximal colonic KEGG metagenome in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. Confidence ellipses (80%) and centroid for each treatment group are also drawn.

Plot loadings of components 1 (Figure 31A) and 2 (Figure 31B) were visualised in barplots. No KEGG classes in both components were influenced by the caprine group (Figures 31A, 31B). Furthermore, most (i.e. 7 out of 10) of the KEGG classes in component 1 were attributed to the ovine group (Figure 31A), whereas the top influences for component 2 were from the bovine group (Figure 31B). The top KEGG class which influenced component 1 was 'imidazolonepropionase', which belonged to the ovine group (Figure 31A). The top two KEGG classes which influenced component 2 were 'stage IV sporulation protein FB' and 'FMN-dependent NADH-azoreductase', both belonging to the bovine groups (Figure 31B). All other KEGG classes influencing component 2 belonged to the ovine group (Figure 31B).

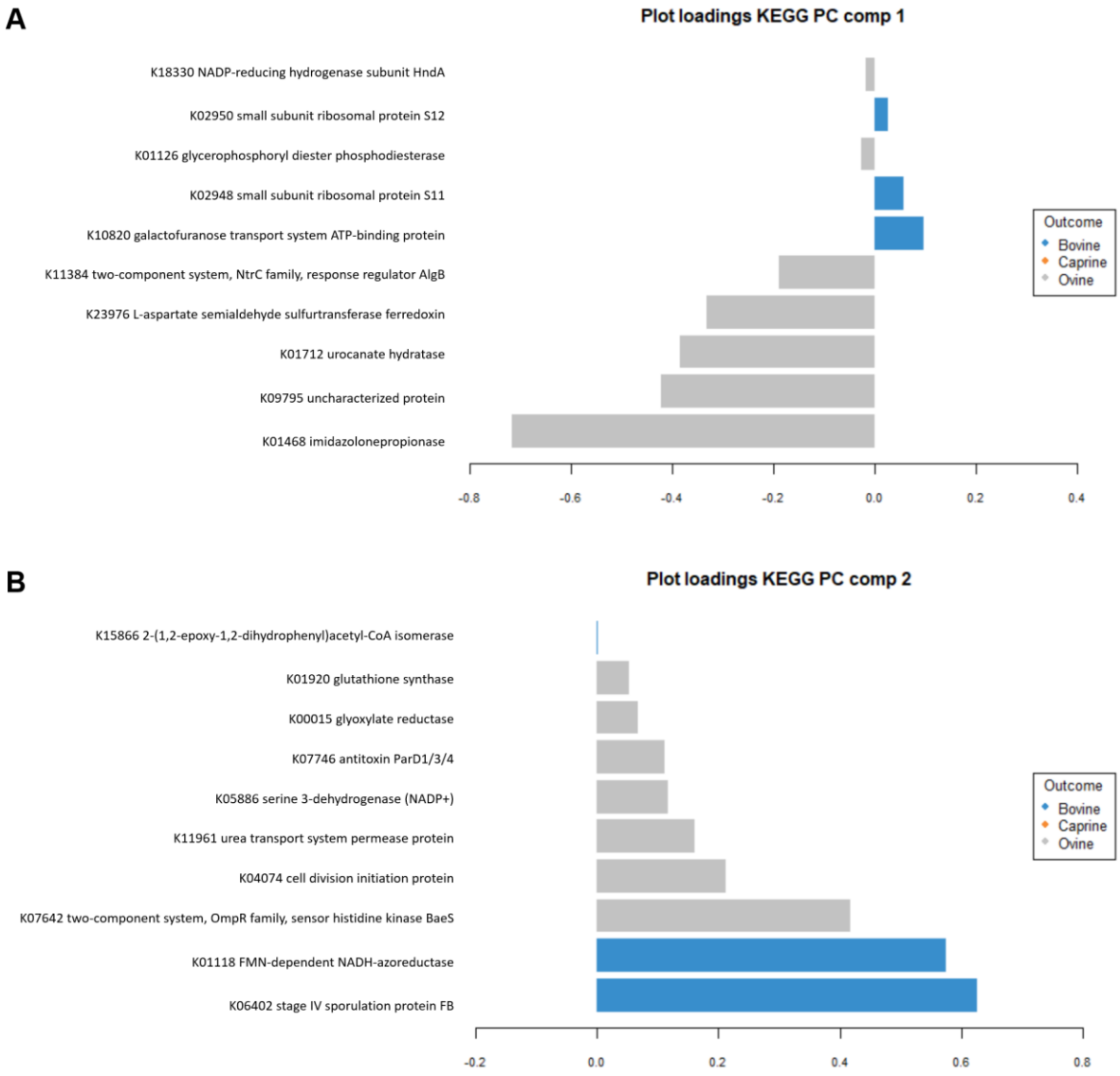

Figure 31. Barplot of top 10 loadings of component 1 (A) and 2 (B) derived from the sPLS-DA plot of proximal colonic metagenome at the lowest KEGG class in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. X-axis shows eigenvalues.

For SEED, again, there was more distinct clustering for each treatment group, but overlaps are evident in the confidence ellipses (Figure 32). This overlap is greater than was observed for KEGG (Figure 30).

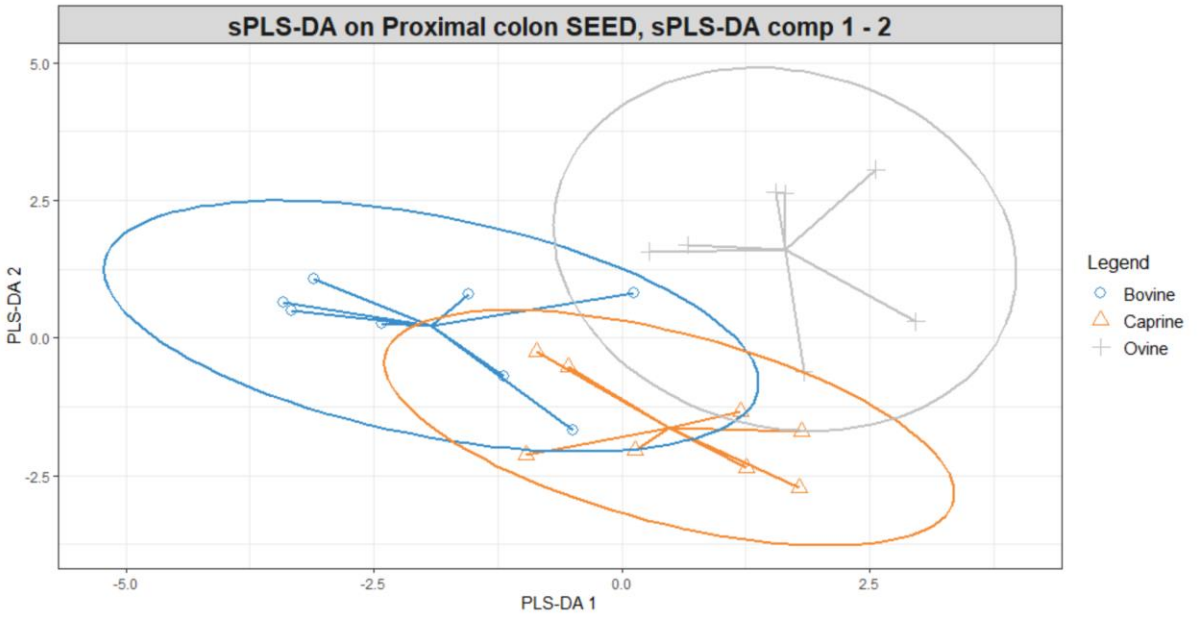

Figure 32. sPLS-DA plot of proximal colonic SEED metagenome in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. Confidence ellipses (80%) and centroid for each treatment group are also drawn.

Plot loadings of components 1 (Figure 33A) and 2 (Figure 33B) were visualised in barplots. For component 1, all but one of the SEED classes were influenced by the bovine and ovine groups (Figure 33A). The top SEED class which influenced component 1 was 'vanillate and syringate utilisation', which belonged to the bovine group (Figure 33A). All SEED classes which inversely influenced component 1 belonged to the bovine group (Figure 33A). For component 2, the top eight KEGG classes were influenced by the ovine group and two remaining classes by the bovine group (Figure 33B). The top SEED class which influenced component 2 was 'resistance to capreomycin and viomycin', belonging to the ovine group (Figure 33B).

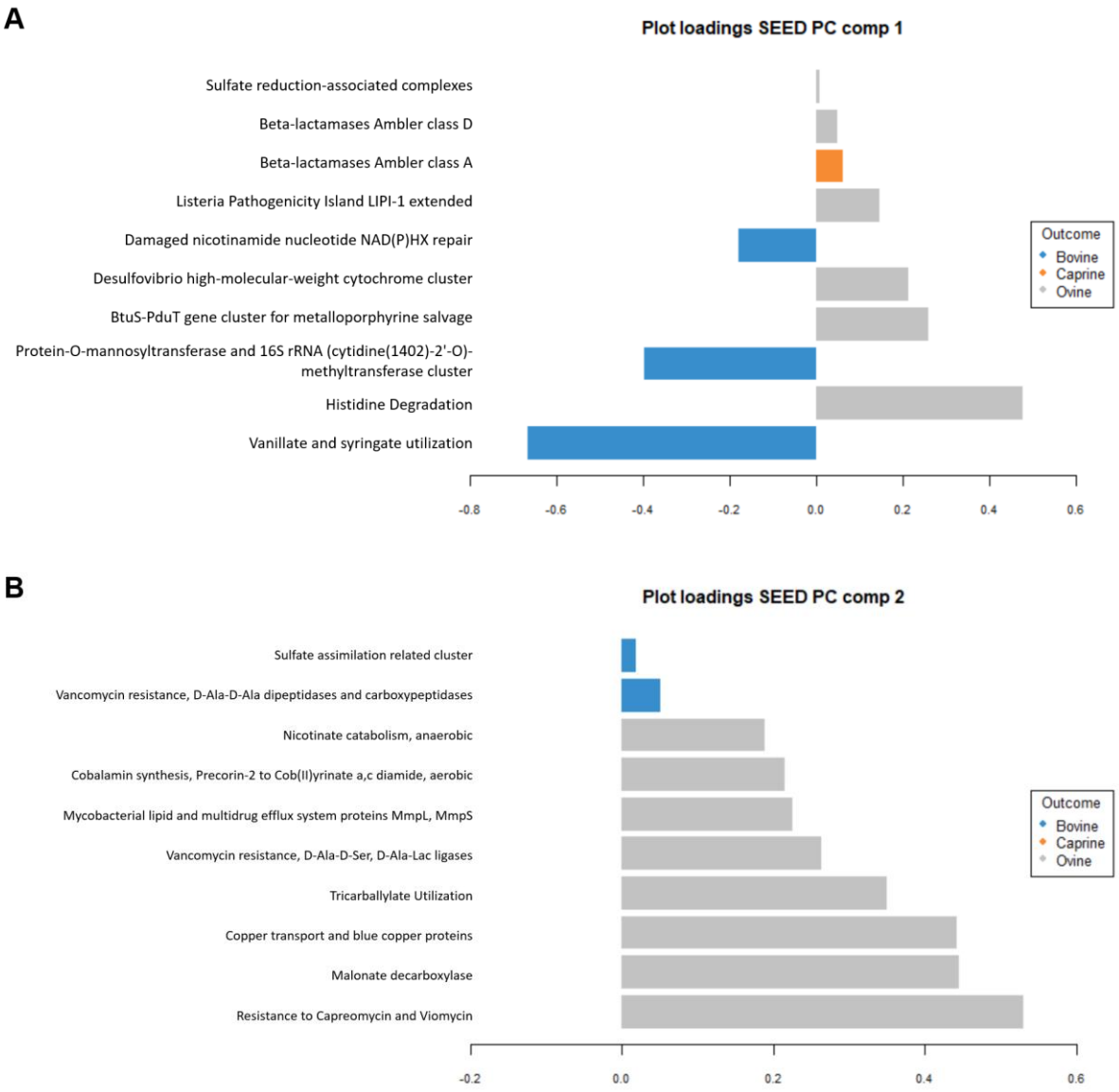

Figure 33. Barplot of top 10 loadings of component 1 (A) and 2 (B) derived from the sPLS-DA plot of proximal colonic metagenome at the lowest SEED class in digesta samples as described previously. Key: Blue – bovine; orange – caprine; grey – ovine. X-axis shows eigenvalues.

Similarly, the gene abundances of the proximal colon samples at the highest KEGG (Figure 34A) and SEED (Figure 34B) orthology classes (Level 1) were plotted in a stacked bar-plot. Again, the most abundant function of the proximal colonic metagenome in digesta samples was related to metabolism, regardless of milk treatments (Figure 34).

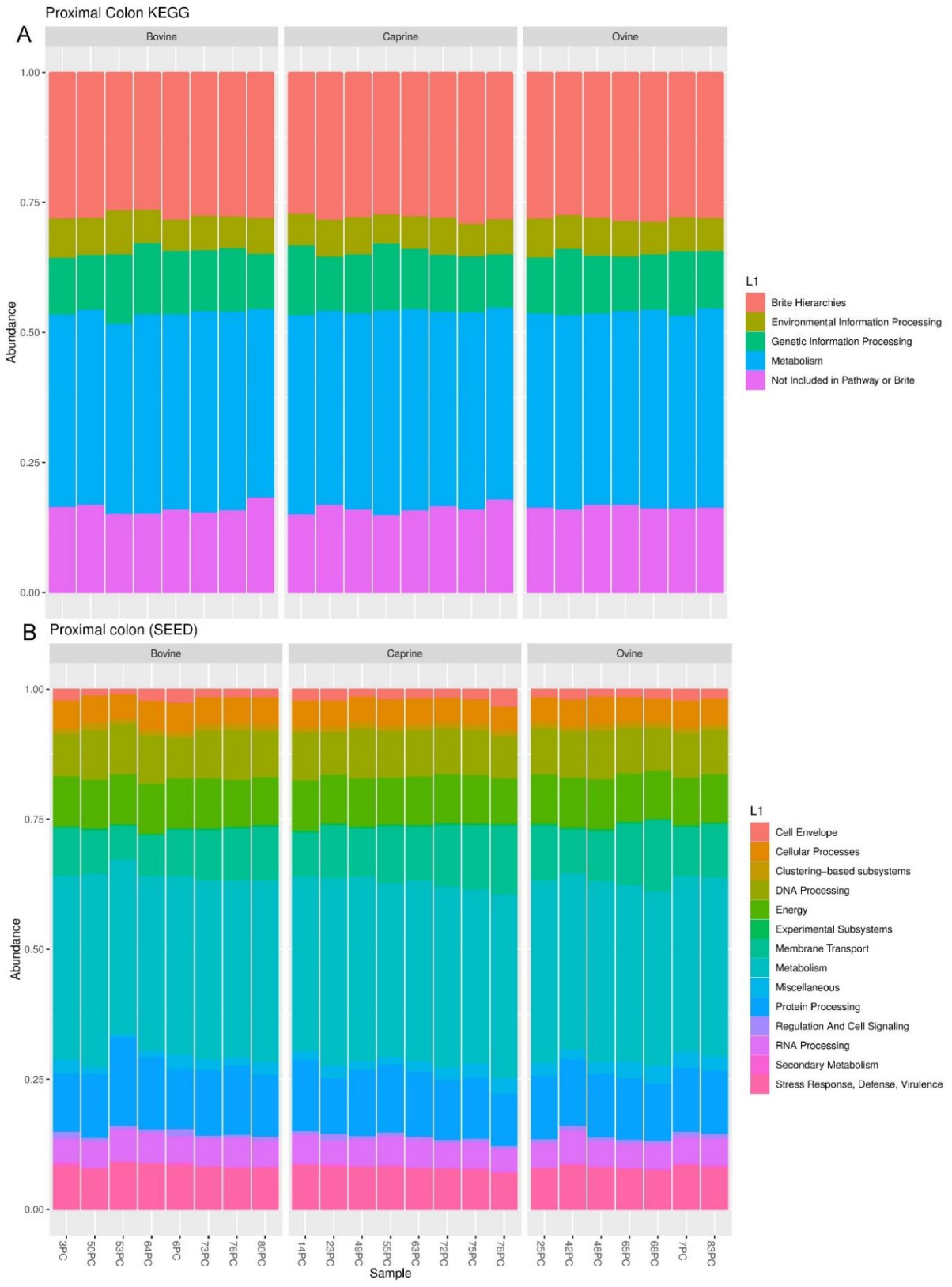

Figure 34. Functional profiles of the proximal colonic metagenome at the highest KEGG (A) and SEED (B) orthology classes (Level 1, L1) in digesta samples as described previously. Colour represents a specific orthology class.

2.5. Discussion

This study is the first to report the composition and gene abundances of the terminal ileal and proximal colonic microbiota of healthy, growing pigs at PND 22 fed exclusively bovine, caprine, or ovine milk for two weeks. Contrary to the stated hypothesis, the milk treatments studied here did not affect the alpha-diversity of the ileal or colonic microbiota. For the beta-diversity, there were no compositional differences between treatment groups at the phylum and family levels, but some minor differences were observed for several genera in both the terminal ileal and proximal colonic microbiota. However, there were no consistent patterns between tissue types or treatment groups. In agreement with the stated hypothesis, the functional potential inferred from the microbial gene abundances of the terminal ileal and proximal colonic microbiota was similar between milk treatments.

Alpha-diversity values ranged from 3.0 to 3.6. These values were dissimilar to the value of 6 reported for pigs from birth to PND 183.[230] Key differences between this study and the study of Wang *et al.* were that pigs were housed individually and ruminant milk was used instead of co-housing and sow milk.[230] Co-housing allows for horizontal transmission of the faecal microbiota between pigs, which may influence the results and increase microbial diversity.[231] Further, the difference in the nutritional composition of the milk treatments and sow milk may explain the lower diversity of the terminal ileal and proximal colonic microbiota in the present study due to differing amounts and type substrates available for the microbes.[232] Furthermore, milk composition evolved to meet the nutritional and physiological needs of their species' neonate and thus impact on their growth and development.[174]

2.5.1. Ileal microbiota

In partial agreement with the stated hypothesis, minor compositional differences at the genus level within the terminal ileum were observed between pigs fed bovine, caprine, or ovine milk. Bacteria belonging to genera *Desulfovibrio*, *Blautia*, *Ruminococcus* or *Prevotella* were not present in pigs fed ovine milk. All four genera were present in pigs fed caprine milk. Only genera *Blautia*, *Ruminococcus*

and *Prevotella* were present in pigs fed bovine milk, with genus *Desulfovibrio* being absent. The aforementioned genera have all been reported to be present in the pig intestines.[233]

Bacteria belonging to the genus *Desulfovibrio* are characterised by their ability to reduce sulphate to hydrogen sulphide during anaerobic respiration.[234,235] A higher prevalence of some species of the *Desulfovibrio* genus has been reported in the faecal microbiota of patients with inflammatory bowel disease when compared with healthy individuals.[236] It has also been posited to play a role in the development of Parkinson's disease.[237] Increased prevalence of *Desulfovibrio* has also been reported to be higher in obese or overweight children as compared with normal-weighted children.[238] However, a large cohort study conducted in Guangdong, China, reported that the presence of *Desulfovibrio* was positively correlated with microbial diversity, increased abundance of beneficial genera and decreased abundance of harmful bacteria.[239] The study highlighted that an increased relative abundance of *Desulfovibrio* goes 'hand in hand' with an increased relative abundance of *Ruminococcus* and *Prevotella*, which was also apparent in this present study.[239] But, this observation of 'aligned' increased relative abundance was not replicated within this present study.

Ruminococcus bacteria are key symbionts of the microbiota in the GIT due to their ability to break down complex polysaccharides into various nutrients for their host.[240] Among these are fatty acids, which convey a multitude of health benefits, including but not limited to reinforcing intestinal barrier function,[241] alleviating inflammation,[144,242,243] regulating immune function,[244,245] and improving metabolism.[80,246] It is unclear why pigs fed ovine milk did not harbour any *Ruminococcus* bacteria in the ileal microbiota, but it may also be linked to the lack of *Desulfovibrio* in the same segments.

Prevotella bacteria has been regarded as a beneficial genus due to its extensive presence in the healthy human body and its rare involvement in infections.[247] *Prevotella* species are dietary fibre fermenters and contribute to polysaccharide breakdown, and their relative abundance is correlated with plant-rich diets.[247] Its abundance has been noted to be higher in populations with fibre-rich diets.[248] However, some strains of *Prevotella* have been suggested to play a potential role as an

intestinal pathobiont.[249] A review by Ley[250] examined the possible beneficial or detrimental effects of *Prevotella* strains on host health and accentuates the importance of understanding its wide genetic diversity, ecology and interactions with other commensal microbes in the GIT to be able to ascertain its potential pathogenic role.[249] Again, it is unclear why in this present study pigs fed ovine milk harboured no bacteria from the *Prevotella* genus in the terminal ileal microbiota but other milk treatments did, and may, once more, be attributed to the lack of *Desulfovibrio* in the terminal ileal microbiota.

Bacteria belonging to the genus *Blautia* have been suggested to possess some probiotic characteristics.[251] A relatively 'new' genus (established in 2008), some species in the genera *Clostridioides* and *Ruminococcus* have been reclassified as *Blautia* following phenotypic and phylogenetic analyses.[252,253] All *Blautia* strains can utilise glucose, but different strains showed different abilities to use sucrose, fructose, lactose, maltose, rhamnose, and raffinose.[251] Furthermore, supplementation of dietary protein and fructooligosaccharides increased the abundance of *Blautia* in rat faecal microbiota.[254,255] Following the standardisation of milk given to constant protein, the amount of lactose present in ovine milk (1.3 g/kg BW) was lower than that of bovine or caprine milk (2.5 g/kg BW) and thus could account for the lack of *Blautia* present in the pigs fed ovine milk due to the diminished food source.

Cluster analyses showed that samples belonging to the caprine and ovine groups formed distinct clusters, whereas the bovine group was more spread out. Furthermore, the overlap of clusters was observed for the bovine and ovine groups, suggesting that these two groups were similar to each other in composition. This finding was contrary to the stated hypothesis that bovine and caprine would be more similar. However, it is important to note that only two caprine samples were available, so there is not much confidence in these results. Additionally, none of the aforementioned genera, namely *Desulfovibrio*, *Blautia*, *Ruminococcus* or *Prevotella*, (identified as significant by ANCOMBC) were one

of the top ten taxa influencing the unsupervised data analyses (PCoA and PCA). However, *Desulfovibrio* was shown to drive the proceeding supervised data analysis (sPLS-DA).

2.5.2. Colonic microbiota

In partial agreement with the stated hypothesis, minor compositional differences within the proximal colon microbiota were observed between pigs fed bovine, caprine, or ovine milk at the genus level. Bacteria belonging to genera *Selenomonas* or *Elusimicrobium* were not present in pigs found in ovine milk, but present in both pigs fed bovine and caprine milk. Bacteria belonging to the genus *Salmonella* were not present in pigs fed bovine or caprine milk, but present in pigs fed ovine milk. The aforementioned genera have all been reported to be present in the pig intestines.[233]

Selenomonas are obligate saccharolytic organisms that break down sugars.[256] The lack of *Selenomonas* in the colonic digesta of the pigs fed ovine milk might relate to their functions. It is worth noting that the volume of ovine milk offered to the pigs was approximately half that of bovine or caprine milk. This adjustment was due to the study design, where the pigs were fed a constant amount of protein, and, therefore, the volume of milk fed was different between treatment groups, as shown in Table 4 (Section 2.3.2). Hence, these pigs had a lower intake of lactose and likely oligosaccharides (not measured here) when fed ovine milk, which might explain the lower abundance of taxa from the *Selenomonas* genus.

Little is known regarding *Elusimicrobium*, a relatively 'recent' genus first cultivated from humivorous scarab beetle larva GIT.[257,258] It grows heterotrophically on sugars such as glucose, fructose and galactose.[257] It is posited that it is absent in pigs fed ovine milk due to the lower availability of lactose (following normalising all milk treatments to constant protein).

Salmonella are a major cause of human morbidity and mortality worldwide, and infections can alter the genomic, taxonomic, and functional traits of the GIT microbiota.[259,260] It was only present in pigs fed bovine or ovine milk, however, at low levels (mean relative abundance of 0.02 and 0.06,

respectively), so it is unlikely this may have impacted the proximal colonic microbiota composition. Also, all pigs were healthy and fed well at the sampling time. The presence of *Salmonella* may be due to some pigs being carriers or contamination.

Unsupervised cluster analyses did not reveal any separation between treatment groups. However, some separation of the ovine group from bovine and caprine groups was observed in the subsequent supervised cluster analyses. This finding is in line with the stated hypothesis, that the bovine and caprine groups would be more similar to one another. None of the aforementioned genera, namely *Selenomonas, Elusimicrobium* or *Salmonella,* (identified as significant by ANCOMBC) were one of the top ten taxa influencing the unsupervised data analyses (PCoA and PCA). However, *Selenomonas* was shown to drive the proceeding supervised data analysis (sPLS-DA).

2.5.3. Functional potential

The data on the relative abundance of genes of terminal ileal and proximal colonic microbiota were used to identify its functional potential (as inferred from the gene abundance data from KEGG and SEED) in pigs fed bovine milk, caprine, or ovine milk for two weeks. As hypothesised, the functional potential of the terminal ileal and proximal colonic microbiota of these pigs did not show any differences between milk treatments. This difference is attributed to the notion of a 'core' microbiota, in that any compositional differences resulting from diet, other microbes can undertake the function of other members in the microbial community.[55,60]

Desulfovibrio, *Blautia*, *Ruminococcus*, *Prevotella*, *Selenomonas, Elusimicrobium* and *Salmonella* genera represent a low proportion of the microbial community of the GIT. Therefore, it is also unclear whether the changes in their relative abundances would have resulted in changes in their metabolism. Equally important, their effects on the metabolism of ileal or colonic tissue or tissues and organs elsewhere in the body, which were not assessed here.

2.5.4. Strengths and limitations

Analysing the ileal and colonic microbiota using shotgun metagenomic sequencing allowed for higher specificity in determining the microbiota composition than the 16S rRNA sequencing used in other studies. Shotgun sequencing also gives better coverage of genomes and more specificity of genes but is less specific to the 16s regions, which amplicon 16s covers well. Furthermore, the multiple quality check steps performed prior to sequencing ensured sufficient quantity and quality of samples for analyses. The ANCOMBC analyses specifically designed to analyse microbiomes provided an insight into any statistical significance, making no distributional assumptions and including bias correction.

Differences were also seen between groups in the terminal ileum. However, the sample size was too small to be confident in these results. The sample size was small due to the difficulty in collecting digesta from the terminal ileum. A larger sample size would be beneficial to compare microbiota composition and the effects of milk formulae on the ileum, a segment involved in absorption (of proteins, lipids and vitamins/minerals) and immune modulation. Additionally, the inclusion of faecal samples may be advantageous, as they can be collected multiple times from the same subjects during the study.

Furthermore, the study design aimed to analyse gastric curd formation during milk digestion and protein is one of the main components that influence this process.[175] Protein concentrations were therefore normalised to be the same across milk treatments to study differences in the rate of gastric digestion based on whole milk structure.[175] Consequently, intakes of all other milk macronutrients (proteins (casein and whey), lipids, carbohydrates (lactose, oligosaccharides) and minerals), and overall volume of milk, were affected. As a result, the effects of milk treatments on the ileal and colonic microbiota composition or functional potential reported here do not reflect the effects of their constitutive compositional differences.

The analysis of the terminal ileal and proximal colonic microbiota was carried out at one timepoint in the later part of early postnatal life development before the pigs normally transitioned to a

complementary diet of milk and solid foods. The measurements were made at PND 22 when pigs are developmentally like a three-month-old human infant. A measurement at one timepoint does not reflect the dynamic nature of the terminal ileal and proximal colonic microbiota as it develops and how concentration differences in nutrients between bovine, caprine or ovine milk might affect or not the establishment of the microbiota.

3. Concluding remarks

In summary, whole bovine milk, caprine, or ovine milk, when exclusively fed to healthy, growing pigs, as a model of the human infant, from PND 7-8 to PND 22, did not affect the predicted gene functions of the terminal ileal or proximal colonic microbiota. However, they differentially affected the relative abundance of taxa from four genera (*Desulfovibrio, Blautia, Ruminococcus* and *Prevotella*) in the terminal ileum and three genera (*Selenomonas*, *Elusimicrobium* and *Salmonella*) in the proximal colon.

The relevance of these findings needs to be interpreted with caution, given that the volume of ovine milk consumed was less than that of bovine milk or caprine milk consumed (this was to ensure the pigs received an equal amount of protein per day). Nevertheless, it is plausible that changes in the relative abundance in these genera with ovine milk would positively influence microbial metabolism and host health.

More research is required to elucidate whether the proposed effects resulted from the modulation of the composition of specific genera of the terminal ileal and proximal colonic microbiota when bovine, caprine or ovine milk is exclusively fed to pigs. Furthermore, longitudinal data are needed to address whether bovine, caprine or ovine milk fed to pigs from two days postnatally until weaning may differentially affect the composition and function of the terminal ileal and proximal colonic microbiota and the development of pigs.

Another important question is whether these effects exist, whether they are associative with or causal to ileal and colonic tissue function, and whether these changes persist during the complementary feeding period and adulthood. Further, if these effects continue to influence other host physiology such as brain and immune development, such knowledge will be important to better understand the role of the terminal ileal and proximal colonic microbiota in the development of the human infant and response to diet.

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Appendix A: Review paper submitted to Frontiers in Microbiology

Effects of early postnatal life nutritional interventions on host immune- microbiome interactions in the gastrointestinal tract and implications for brain development and function

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"Effects of early postnatal life nutritional interventions on immune-microbiome interactions in the gastrointestinal tract and implications for brain development and function"

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Abstract

The gastrointestinal (GI) microbiota has co-evolved with the host in an intricate relationship for mutual benefit, however, inappropriate development of this relationship can have detrimental effects. The developing GI microbiota plays a vital role during the first 1,000 days of postnatal life, during which occurs parallel development and maturation of the GI tract, immune system, and brain. Several factors such as mode of delivery, gestational age at birth, exposure to antibiotics, host genetics, and nutrition affect the establishment and resultant composition of the GI microbiota, and therefore play a role in shaping host development. Nutrition during the first 1,000 days is considered to have the most potential in shaping microbiota structure and function, influencing its interactions with the immune system in the GI tract and consequent impact on brain development. The importance of the microbiota-GI-brain (MGB) axis is also increasingly recognized for its importance in these developmental changes. This narrative review focuses on the importance of the GI microbiota and the impact of nutrition on MGB axis during the immune system and brain developmental period in early postnatal life of infants.

Keywords

Diet; Gastrointestinal microbiota; Gastrointestinal-brain development; Microbiota-Gastrointestinal-brain axis; Early postnatal life

1. Introduction

The gastrointestinal (GI) tract represents one of the largest sites of interaction between environmental factors, the host, and the microbiota [1]. The GI microbiota, a collection of microorganisms including bacteria, archaea, and eukarya, resides within the GI tract (mouth to anus) and has co-evolved with the host to form intricate and mutually beneficial or detrimental relationships [2]. Benefits include immune development through strengthening and maintaining epithelial integrity of the small and large intestine, with protective effects against pathogens through physical exclusion. The GI microbiota may also directly influence host metabolism. For example, it directly breaks down foods and food remnants as they progress through the GI tract, altering gene expression of the mucosal cells to facilitate the breakdown of foods, and producing enzymes that are not encoded in the human genome that perform digestion of food remnants [3; 4; 5].

There is a critical time window for microbial colonization in early postnatal life, 'setting the scene' for continued GI, immune, and brain development [6]. Disruption of the colonization process or microbiota composition (i.e., dysbiosis) can negatively affect the host, and has significant consequences for the immune system and brain development, thereby impacting cognitive function and behavior [2; 7]. The bidirectional communication pathways between the GI tract and brain are termed the microbiota-gut-brain (MGB) axis; these pathways have been increasingly studied as a potential target for therapeutic applications in preventing or treating a disease or reducing disease symptoms [8]. However, the mechanisms underpinning the MGB axis and their roles in the critical window of microbiota colonization during the first 1,000 days of postnatal life warrant more research.

Nutrition is among the foremost determinants of, and directly affects, GI microbiota colonization, composition, and function [9]. Dietary diversity is positively correlated with greater microbial diversity, due to the increased availability of metabolic substrates for the large intestinal GI microbiota [10]. During the first 1,000 days, nutritional intervention is considered to have the most potential in shaping microbiota structure and function and their consequent impact on the GI, immune system, and brain development [6; 11].

This narrative review summarizes current knowledge surrounding the importance of the microbiota for development of the GI tract, immune system, and brain during early postnatal life. The

review will then discuss the impact of nutrition has on the GI microbiota and its potential role in developing these systems in early postnatal life and highlights new areas of research.

2. Microbiota colonization of the gastrointestinal tract and its effect on host-microbe interactions

While for some time there has been debate over whether the infant is born with a sterile GI tract, recent evidence supports the hypothesis that bacterial DNA are not present within the placenta and almost all microbial DNA detected accounted for by either the introduction during delivery (caesarian) or laboratory reagent contamination [12].

Following birth, there are broadly four phases of microbial colonization of the GI tract: 1, first exposure as the infant is born; 2, continuous stimulation of the microbiota as a result of oral feeding; 3, additional stimulus of the microbiota with the introduction of solid foods (weaning transition); and 4, stabilization of the composition into an adult-like microbiota [2; 13]. These phases are summarized in Figure 1, which also shows the parallel development of the infant's GI tract, immune system, and brain. These various phases of microbiota colonization are affected by different factors: mode of delivery, infant feeding methods, gestational age, weaning duration, frequency and severity of infection, and antibiotic exposure [14]. The composition can therefore fluctuate in response to such factors but stabilizes at around three years of age.

Microbial distribution and diversity also increase along the GI tract due to nutrient, chemical and oxygen availability from the proximal to the distal portions. There is a more acidic, oxygenic, and antimicrobial environment in the small intestine than in the colon, where pH is closer to neutral and the colonocytes rapidly consume oxygen [15]. Here, the obligate anaerobes outnumber aerobic and facultative anaerobic bacteria by 100- to 1,000-fold [15].

Successive pregnancies or maternal parity is also a known risk factor in infant health such as growth, mortality, and even premature birth making parity a significant contributor to the microbiome during pregnancy and infant health [16]. Because of this, parity should also be considered when designing microbiome studies involving pregnancy and infants.

2.1 The microbiota-gut-brain axis

The association of the microbiota with the host, known as the MGB axis, involves bidirectional signaling pathways, and encompasses several organ systems: GI microbiota, enteric nervous system, endocrine system, immune system, central nervous system, autonomic nervous system, hypothalamic-pituitary-adrenal axis, and vagus nerve [17; 18; 19]. Communication within the MGB

93 axis is regulated using neural, endocrine, immune, and metabolic pathways [11; 17]. It involves several
94 neurotransmitters and metabolites (e.g., essential vitamins, secondary bile acids, amino acids, and
95 organic acids) which concentrations must be balanced to maintain homeostasis [17; 20].

96 Our understanding of the role that the GI microbiota plays within the MGB axis has advanced
97 through research with animal models. Whilst possible, clinical studies performed in humans are
98 difficult, often limited to the collection of fecal samples as a representative of the microbiota
99 composition of the large intestine [21]. Non-human primates are most comparable to humans; they
100 share physiological, metabolic, and genetic similarities [22]. However, their ethical concerns,
101 maintenance cost, and lifespan limit their use for research [23; 24; 25; 26; 27].

102 Fecal microbiota transplants in humans, which involves the transplant of the fecal microbiota
103 from one individual to another, have also been used to study the MGB axis [28; 29]. When successful,
104 the recipient's microbiota compositional signature or profile is more like the donor than the recipient
105 [29]. It has been observed in several studies that behavioral phenotypes, for example depression or
106 anxiety, may be transferred along with the microbiota [30; 31; 32; 33]. Infants may benefit as well. In
107 a proof-of-concept study, the transfer of maternal fecal microbiota was shown to restore normal
108 microbiota development in cesarean-born infants [34].

109 Of research involving humans, it has been reported that the MGB axis involved in infant
110 development connects general neurocognitive development, socio-emotional behavior, and physical
111 brain structure where function is assumed from neuroimaging [35]. Substantial variation exists across
112 studies, however, most likely due to the methodologies, sample sizes, DNA sequencing methods, and
113 even the statistical models applied. Standardization of sequencing methods and carrying out whole
114 metagenome analyses are required to improve our understanding of the role of the gut microbiome
115 in brain development during those early postnatal development stages [35]. Further research is
116 needed in infants using methods to assess the role of the MGB axis in longitudinal brain development
117 and behavior.

118 Researchers have applied other methods of studying the MGB axis in mice which includes the
119 use of antibiotics to alter the microbiota composition to elicit behavioral or cognitive changes [36; 37;
120 38]. In another recent study, changes in the microbiota of adult mice brought about with antibiotics
121 or germ-free conditions resulted in behavioral changes, specifically, deficits in fear extinction learning
122 [36]. This behavior deficit could be restored through by reestablishment of the microbiota suggesting
123 that the microbiota produce compounds that are able to directly affect brain function. It may be also
124 that probiotics can prevent brain injury through blocking the ability of damaging molecules to reach
125 the brain via the MGB axis [37]. Immune systems work in concert. For example, neonates also develop

maturation of their dendritic cells, which is brought about through exposure to the microbiota. The dendritic cells produce interleukin (IL)-12 which accumulates in the environment and through a mechanism where IL-12 offsets the bias towards Th1, thereby promoting Th1 differentiation to Th2 [38].

Pigs are a more suitable and preferable choice over rodents for research focusing on early postnatal life development of the human infant. Pigs and human infants share similar scaled GI and brain developmental timelines as compared to rodents [39]. In general, the porcine brain is one-tenth the total volume of the human brain throughout its lifespan with one month of human growth being approximately equivalent to one week of piglet total brain growth. This is in contrast to rodent brains, which are much smaller, grow and develop with a different trajectory, and would often require pooling tissue to analyze its components [39]. Furthermore, the precocial nature of piglets allows for behavioral and cognitive assessments to be performed immediately after birth, which is impossible in either rodents or humans [39; 40]. Additionally, most knowledge in immune ontogeny in early postnatal life has stemmed from studies with germ-free rodents, which provide results that are not readily translatable to humans. Pigs are, again, an improved animal model as they share greater similarities with humans in terms of anatomy and function of the immune system (e.g., pigs possess tonsils whereas rodents do not) [41].

Pigs are also the animal model of choice in terms of digestive and associated metabolic processes, as their nutritional requirements when compared with other non-primate animal models are more closely aligned to those of human (infants) [42]. Cannulation is also possible for repeated, stress-free digesta sample collection to assess nutrient digestibility and kinetics over the lifetime [43; 44]. Therefore, the use of pigs in early postnatal life developmental research allows for the determination of the critical time windows of GI, brain, and immune development that may be sensitive to nutrition intervention.

2.2. Development of the immune system

Newborn infants need time to develop and mature their innate and adaptive immune systems. There are some transfers of maternal immunity both *in utero* and through breast milk. These processes help to provide immunity to the infant in the first days through transfer of both secretory immunoglobulins and 'milk leukocytes', which produce molecules that migrate to the GI and respiratory tracts [45; 46; 47]. Over time, the components of breast milk change from a primarily protective to a nutritional role [2; 48].

The maturation of the immune system requires the GI microbiota, the absence of which impacts all aspects of immune system development and function [49]. Improper development brings about several immunological defects, including increased susceptibility to infections and altered immune homeostasis [50; 51; 52]. It has been observed that mice treated with antibiotics, or gnotobiotic mice colonized by a limited defined consortia of bacteria, showed impaired microglia maturation and immune response upon bacterial stimuli when compared with their conventional counterparts [53]. Training of the infant immune system is also required for optimal GI function, including vascular supply, epithelial healing, nutrient absorption, and protection from infection.

There is an interface between innate and adaptive immunity, where T or B cells are recruited and work at protecting intestinal mucosal from the resident microbes. The immune system includes the Toll-like receptors (TLRs) that recognize the presence of microbes from their DNA to their surface molecules to the specialized T cells. For a review of 'the interface between innate and adaptive immunity' see Hoebe et.al. [54].

Innate immunity includes the mucosal-associated invariant T (MAIT) cells, abundant in humans and a key immune system component. MAIT cells are atypical T-cells with a limited response repertoire activated by riboflavin-derived molecules (rather than peptides), which are presented through the major histocompatibility complex (MHC) class I protein MR1 [55]. MAIT cells are absent in germ free mice and become more abundant in microbial-challenged mice, so it is highly likely that exposure to and association with microbes in the GI tract is responsible for their development and maturation [55; 56]. Similarly, invariant natural killer (NK) T cells are abundant in healthy infants and have a role as 'cytotoxic effectors' and regulation of the adaptive immune system [57; 58].

Immunoglobulins are also part of the innate and adaptive immune response and are critical to the infant's ability to specifically recognize and bind to antigens, which facilitates their destruction. There are five main classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE), characterized by the type of heavy chain within their structure, resulting in differences in their function and type of immune response elicited by each molecule. Secretory IgA (sIgA) is transferred through maternal breast milk to the infant where it protects from infection and is critical for homeostasis of the microbiota not only through encouraging colonization but through influencing the microbiota gene expression [59; 60; 61; 62]. Maturation of IgA and IgG requires B cell class-switching, and this process does not mature until around six months of age. IgE may also be produced through B cell isotype switching at mucosal sites and abnormally high plasma IgE levels have also been observed in germ-free mice compared with conventionally reared mice. A study on this concluded that a sufficient microbial stimulation during early postnatal life is required to maintain baseline IgE levels [63]. Additionally, it has been

190 demonstrated that the 'allergy phenotype' is transferrable via transplantation of the GI microbiota
191 [13; 64]. Germ-free mice are inherently susceptible to anaphylactic responses to food, quantified by a
192 drop in body temperature. The colonization of these mice with the microbiota from healthy infants
193 protected the mice from anaphylactic responses, but not when colonized using the microbiota from
194 infants suffering from bovine milk allergy [64; 65].

195 Research in rodents have shown that appropriate innate immune responses are also required
196 for nutrient absorption and metabolism. Shulzhenko *et al.* identified inter-connecting regulatory
197 signaling networks which balance metabolism and the innate defensive mechanisms in epithelial cells
198 [66]. If IgA concentrations are altered, these networks become unbalanced and may cause irregular
199 upregulation of certain pathways (e.g., innate immunity), while downregulating others (e.g., lipid
200 uptake and metabolism) [66]. Furthermore, bacterial fermentation of indigestible dietary fibers
201 produces organic acids in the colon [67]. Organic acids promote intestinal barrier integrity, mucus
202 production, and supporting a tolerogenic response over inflammation [2; 67; 68; 69].

203 Adaptive immunity involves the maturation of T and B cells which begin their development
204 while still *in utero* [70; 71; 72; 73]. Infant responses to antigen by T and B cells are known to be weaker
205 than in adults in most cases except in some vaccines and pathogens [74]. It is important to
206 acknowledge that much of our understanding early postnatal life immunity in infants has relied on
207 murine models even though they differ substantially in ontogeny as reviewed by Semmes et al. [75].

208 *2.3. Role of the gastrointestinal microbiota in brain development*

209 Brain development begins *in utero* and continues into adolescence [76], as shown in Figure 1.
210 Major events of brain development in early postnatal life include synaptogenesis, myelination, and
211 synaptic pruning. The GI microbiota is known to affect or be associated with these events, as well as
212 influence neural development, cognition, and behavior. Cognitive functions, including learning
213 capacity and memory, are closely linked with the GI microbiota [77]. Cognitive function encompasses
214 the life-long process of learning, which includes both long- and short-term processes [78]. Cognitive
215 impairment has been noted in individuals with GI or neurological disorders [78; 79]. Several human
216 studies have been published already, correlative, linking the microbiome with brain function and a
217 summary of these and the findings has been reviewed recently [6]. One recent study looked at the
218 fecal microbiota profiles of one-year-old infants and correlated these with regional brain volume data
219 and Mullen Scales of Early Learning (indicator of cognitive performance) at two years of age, finding
220 differences [80]. Although brain volume was not different between groups, the fecal microbiota alpha
221 diversity differed between age groups [80]. A key finding in that study was that highest level of
222 cognitive performance could be predicted by the *Bacteroides* genus while the lowest level of

performance was predicted by the *Faecalibacterium* genus. While there was a difference in cognitive performance depending on microbiome diversity, there was no evidence of a 'cognitive damage' microbiota profile or nor any cognitive impairment in either age group [80].

Studies using germ-free animal models or antibiotic-induced dysbiosis have been used to demonstrate that without a 'normal' GI microbiota, working and spatial memory are negative influenced [79; 81]. For instance, elevated hippocampal levels of serotonin [82] and brain-derived neurotrophic factor [83; 84] were linked with behavioral changes between germ-free and conventional rodents, such as increased depressive-like and decreased anxiety-related behaviors [85; 86; 87; 88]. The decreased anxiety-like behavior in germ-free mice was observed when subjecting the mice to various tests that measure the natural aversion of rodents for open and elevated areas and their natural, spontaneous exploratory behavior in novel environments. This cautious versus exploratory behavior must be balanced to ensure survival of the individual for procreation. Whilst decreased anxiety may be advantageous, this may lead to an increased chance of predation and therefore decrease their likelihood of survival. The exhibited aberrant behavior persisted following the colonization of these germ-free mice with a conventional microbiota. These findings show that the GI microbiota plays a role in developing stress pathways and a critical time window exists for reconstitution of the microbiota to normalize behavior [85; 86; 87; 88]. Similar findings have been reported where a critical time window of colonization exists to avoid negative behavioral changes in adulthood [89; 90]. Furthermore, a study by Bercik *et al.* showed that behavioral changes are transferable following transplantation of the GI microbiota [30].

The GI microbiota is also linked to changes in neurogenesis [78; 91; 92; 93]. In a recent review on how the microbiota composition impacts neurogenesis, possible strategies for using the microbiome to treat neurological disorders is discussed but the mechanisms for microbiome inhibition or promotion of neurogenesis are still not understood [94]. The primary mode of communication from the GI microbiota to the host's central nervous system is achieved through immune or endocrine mechanisms [17]. These mechanisms are often mediated by microbially-derived molecules such as organic acids and tryptophan metabolites [95; 96; 97]. For example, some 90% of serotonin required for mood, behavior, sleep, and several other functions within the central nervous system and GI tract is produced by the intestinal microbiota [98; 99]. These metabolites signal enteroendocrine and enterochromaffin cells, which in turn may act directly or indirectly on the central nervous system [17]. Direct signaling must overcome obstacles such as the epithelial barrier and immune system in the GI tract or blood-brain barrier to exert its effects on the brain [96; 100; 101]. Indirect signaling may induce responses in the central nervous system through long-distance neural signaling by vagal and/or spinal afferents [102; 103]. Alterations to these signaling pathways within the MGB axis have been implicated

in the pathogenesis and pathophysiology of both functional GI and neurological disorders [104; 105]. An approach may be developed for prevention and treatment by targeting specific mechanisms through which the GI microbiota interacts and contributes to these disorders [104; 105; 106; 107].

Organic acids produced primarily by the colonic microbiota have been observed to exert effects on blood-brain barrier permeability [108]. The three most abundant organic acids are the short-chain fatty acids acetate, propionate and butyrate [109]. Acetate produced in the intestine crosses the blood brain barrier and is taken up by the brain where it is incorporated into the hypothalamus and eventually has a role in appetite regulation [110]. Propionate has an effect on the regulation of the sympathetic nervous system, and both propionate and butyrate affect intracellular potassium levels [111], and also regulate the expression of tryptophan hydroxylase, which in turn is involved in the biosynthesis of serotonin [112]. For a review on the role of the organic acids in the MGB axis see Silva et al. [67].

The GI microbiota plays a role in the normal development and regulation of the hypothalamic-pituitary-adrenal axis. This axis is a major component of the homeostatic response that mediates the effects of stressors by regulating many physiological processes. Thus, the GI microbiota can influence the host's stress reactivity and anxiety-like behaviors [113; 114]. For example, maternal separation stress models, e.g., where young are separated from their mothers to stimulate a stress response, have been used to assess how acute and/or chronic stress affects the mouse pups. Stress has resulted in memory dysfunction in germ-free rodents, attributed to altered brain-derived neurotrophic factor expression levels [115]. This factor regulates several aspects of the brain, and its altered expression can lead to downstream effects on cognitive functions and intestinal muscle repair, regeneration, and differentiation [116]. Germ-free animals also exhibited decreased anxiety and increased stress response with augmented levels of adrenocorticotropic hormone and cortisol [90; 117; 118; 119; 120]. Following recolonization of the GI tract with a conventional microbiota, normalizing the hypothalamic-pituitary-adrenal axis occurs in an age-dependent manner [89]. Reversibility of the increased stress response is achievable only in germ-free mice aged less than three weeks, supporting the notion of a critical period during which the plasticity of neural regulation is sensitive to input from the GI microbiota [89].

Stress during early postnatal life has been shown to have long-lasting effects, including altering the MGB axis. A study by O'Mahoney *et al.*, using the maternal separation rat model, showed altered composition of the fecal microbiota following early postnatal life stress as well as altered behavior and systemic immune responses compared to a control group [121; 122]. Furthermore, basal adrenocorticotropic hormone levels and increased anxiety-like behaviors were higher in the stressed

groups when compared with the control group [123]. However, following a subsequent stressor, adrenocorticotropic hormone levels were lower in the stressed groups and were accompanied by altered neurotransmitter levels, indicating that the stressor had detrimental effects on regular stress responses and induced abnormal behaviors [113; 123; 124]. Psychological stress can directly affect the integrity of the tight junction proteins responsible for barrier integrity for the intestine and the blood brain barrier and loss of this integrity is also correlated with microbiota dysbiosis [125]. Chronic stress can also create a loop that affects memory through a feed-forward loop mechanism leading to depressive disorders as shown in Japanese quails [126]. In this animal model study, Japanese quails experienced induced stress with their microbiota transferred to unstressed quails. Again, the *Bacteroides* genus was implicated with the *Alistipes* genus showing increased abundance in the stressed group and this stress response was transferred through the microbiota to the unstressed recipient [113; 123; 124; 126].

3. Early postnatal life nutrition and the microbiota-gastrointestinal-brain axis

The microbiota is susceptible to modulation by external factors prior to stabilization of its composition at approximately two to three years of age. Among these, infant diet has been identified as a major contributor to GI microbiota development in early postnatal life. As such, the effects of infancy diet (namely formula vs breast milk) on early postnatal life development have been well documented [127; 128]. Comparably, less is known about the effects complementary feeding has on infant microbiota composition [2; 129]. Given the dominating influence diet and nutrition has on microbiota composition, and the involvement of the microbiota in regulating immune and brain development, gaining a deeper understanding of the potential microbe-mediated host effects of feeding mode in early infancy is needed[130]. Also, the complementary feeding period (6–24 months of life) coincides with a critical period in microbiota development, transitioning away from the influence of a milk-based diet [2; 129]. The infant microbiota composition stabilizes and resembles an adult-like microbiota at around three years of age and attempts at modulation are likely to be more successful if they are conducted before this or early in this period to elicit any beneficial downstream effects.

3.1. Early infancy diet

Breast milk is the recommended first nutrition for the infant, providing all necessary nutrients to support growth and development, as well as passive immunity to protect against infectious diseases during infancy. After lactose and lipids, human milk oligosaccharides (HMO) are the third most abundant component of breast milk. These comprise short saccharides composed of five monomeric

building blocks (glucose, galactose, fucose, N-acetylglucosamine, and sialic acid), of which over 200 different structures have been identified. These oligosaccharides are responsible for selectively promoting the growth and function of beneficial bacteria As infants lack the necessary enzymes to digest HMOs, the molecules pass into the large intestine and function as a carbon source for commensal bacteria [13; 131; 132; 133], promoting and stimulating the growth of specific bacterial groups such as *Staphylococci* [134; 135], and from genera *Bifidobacterium* [136; 137], *Streptococcus*, *Lactobacillus* [138] and *Bacteroides* . Only bacteria such as *Bifidobacterium longum* subspecies *infantis* lineage harbor genes to express all enzymes required for degrading and utilizing HMOs [139; 140; 141] However, other bacteria may cleave and utilize specific components of HMOs [139; 141]. HMOs have been attributed to the two-fold increase of *Bifidobacterium* cells in breastfed infants compared to formula-fed infants [14]. Some bacteria, including *Bacteroides fragilis*, *Bifidobacteria infantis,* and *Lactobacillus acidophilus*, stimulate endogenous production of sIgA, activation of regulatory T cells and anti-inflammatory molecules – all necessary for host homeostasis [142; 143; 144]. Lactoferrin, another component in breast milk, also encourages the proliferation of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* genera [145].

Breastmilk has also been demonstrated to provide passive protection and stimulate the development of the infant's immune system [146]. For example, polymeric IgA and defensins can interfere with pathogen attachment and uptake [144], while *n-3* fatty acids [147] and transforming growth factor-β [148] can activate enterocytes to produce anti-inflammatory cytokines, and lactoferrin can interact with the GI tract and promote immune homeostasis [144; 149].

Comparison of the intestinal microbiota in formula and breast-fed infants showed that at around forty days, those fed exclusively with formula had greater alpha diversity while both breast-fed and formula-fed infants were colonized predominantly with *Bifidobacterium* species and members of *Enterobacteriaceae* family [150]. While the diversity in the breast-fed infants was lower than formula-fed at day 40, it increased by four months and diversity was similar between formula and breast-fed infants. Lower diversity in breast-fed infants was most likely due to the breast milk which requires specific bacteria capable of degrading the oligosaccharides that are in the milk. Although many infant formula products are supplemented with prebiotics such as fructo-oligosaccharides and/or galacto-oligosaccharides, these are not as selective as HMOs since they can be utilized by most *Bifidobacterium* species [151] and stimulate the growth of various *Lactobacillus*, *Streptococcus*, and *Bacteroides* species [152; 153]. Comparison of metabolic profiles of infants fed either exclusively formula or breastmilk have also confirmed that the metabolic capabilities of the microbiota are primarily proteolytic and saccharolytic, respectively [154; 155].

3.2. Weaning and complementary feeding

Over the course of infancy, a point is reached where milk-based feeding is no longer adequate to cover the nutritional requirements of the infant. Therefore, supplementation with additional foods is required. The introduction of solid foods and the progressive reduction of milk-feeding lead to major GI microbiota compositional and functional changes. Bacteria belonging to families *Bifidobacteriaceae* and *Enterobacteriaceae* are decreased after weaning [156; 157], and any compositional differences between breastfed or formula-fed infants slowly decrease [14; 145; 158].

Pigs also produce milk oligosaccharides (pMOs); Twenty-nine were described in 2010 and were found to be abundantly sialylated making them more similar to bovine than human [159]. However, some pMOS are fucosylated, are much more abundant in pigs than in bovine (9% versus 1%) which suggests that pMOs are actually more closely like human than bovine milk and therefore are also influencing the microbiota [160]. In a neonatal piglet model, the fecal microbiota composition stabilized ten days post-weaning [161]. The sudden change from high-fat, low-carbohydrate milk (pre-weaning) to a high-carbohydrate, low-fat feed (weaning and onward) resulted in a drastic change of available nutrients to the commensal bacteria [162]. The predominant genera post-weaning are microbes efficient in degrading dietary fibers and producing organic acids [161], resulting in a microbiota composition more adult-like [161; 162; 163]. Chen *et al.* observed a shift from a high prevalence of *Lactobacillus* and *Bacteroides* genera, to *Roseburia, Paraprevotella* and *Blautia* genera, post-weaning [161]. Furthermore, Firmicutes and Bacteroidetes remained the most abundant phyla pre- and post-weaning [161; 164; 165]. Another study supported these findings, reporting *Bacteroides* as the most abundant genus in nursing pigs (pre-weaning), with *Prevotella* and *Lactobacillus* genera enriched in weaned pigs [162; 163].

Interventions with probiotics to improve or maintain good health in early postnatal life have gained much popularity in recent decades [166]. Some infant formula has been designed to include probiotics, for example *Bifidobacterium* and *Lactobacillus* species, to mimic the composition of human breastmilk. However, probiotic-supplemented infant formula contains a much higher concentration of these probiotic strains when compared with breastmilk [167]. Furthermore, not all probiotics are functionally equal, as the effects obtained from one strain cannot be assumed to be replicable with another strain, even if they belong to the same species [168]. Even if they are not equal, probiotics have been demonstrated to be safe and effective across multiple studies involving infants. For example, supplementation with probiotics can increase the microbial metabolism of milk oligosaccharides in infants while also reducing intestinal inflammation [169]. In another recent study, probiotics demonstrated efficacy against the development of antibiotic resistance in preterm infants

388 [170] and in another, probiotics were shown to cause no adverse effects in vulnerable premature
389 infants and decrease the risk of necrotizing enterocolitis [171].

390 Cognitive impairment is sometimes alleviated through probiotic administration [172; 173; 174].
391 For example, administration of *Lactobacillus helveticus* improved the stress response and cognitive
392 dysfunction induced by chronic stress in rats [172], and *Bifidobacterium longum* strains were observed
393 to be effective in improving memory [175; 176]. Early postnatal life probiotic intervention has also
394 been observed to reduce sepsis and allergy, as well as having a possible role in reducing the risk of
395 neuropsychiatric disorders such as autism spectrum disorder and attention deficit disorder [177; 178;
396 179; 180; 181]. There is also evidence that a combination of *Streptococcus thermophiles* and
397 *Bifidobacterium* genus can be effective in preventing the onset of diarrhea in children following
398 antibiotic treatment [182]. Four *Lactobacillus* strains isolated from breastmilk protected against
399 infection in a mouse model, with potent antimicrobial properties [183]. Thus, cultivating microbes
400 from human breastmilk may also prove to provide good probiotic candidates for further research and
401 development.

402 The application of prebiotics and probiotics in improving health in early postnatal life is
403 promising, but the timing of administration, the quantity administrated, the effect of different strains,
404 combination of strains, engineering, and safety must be carefully considered and continually
405 researched to fully understand how they modulate the GI microbiota composition and exert their
406 effects.

407 There is a developmental aspect of the growing infant where multiple maturation events are
408 proceeding in the brain; in one direction is synaptogenesis, microglia maturation, with targeted
409 synaptic pruning and myelination. Meanwhile, in the immune system, T and B cells are maturing,
410 innate immune cells are being trained through exposure to the environment, and the immune system
411 is learning to differentiate self from non-self (i.e., foreign). Similarly, in parallel, the microbiota in the
412 GI tract is also maturing, colonizing the intestine, metabolizing nutrients, and releasing microbial
413 products that may be immune modulatory or in the case of butyrate, an energy source for enterocytes
414 lining the intestinal epithelium. The vagus nerve sits right at that junction of the MGB axis and is an
415 integral part of the communication between systems. It accesses the entire digestive wall yet does
416 not cross the epithelial layer and it senses and responds to the signals that come from the microbiota
417 transported across the epithelial layers by host entereroendocrine cells [184]. If this communication
418 system the vagus nerve was targeted, it may be able to affect and restore the homeostasis of the MGB
419 axis [185].

4. Future perspectives

The mutualistic relationship that exists between the host and microbes begins at birth and shapes both host health and microbiota composition and function. The development and maturation of the GI tract, immune system, brain, and microbiota are in turn influenced by host genetics and exposure to the environment (e.g., diet, delivery mode, feeding methods, weaning, infection, and antibiotics). There are important interactions between the development and maturation of the immune system that drive the establishment and maturation of the microbiota in the GI tract and potentially affect the brain development and function and associated cognitive behaviors in infants.

The implications of the microbiota and immunological findings discussed in this review for pregnant women, mothers, infants, infant nutrition policy makers, formula manufacturers, and health-conscious consumers are important aspects to consider. The effects of the microbiota on the mental and cognitive state of the infant cannot be ignored. Development and maturation are staged and interdependent processes, with a narrow window of opportunity where nutrition can modulate the microbiota for beneficial effects to be conferred to the infant. Appropriate nutrition might encourage more beneficial bacteria within the microbiota to flourish however, such intervention is a balance between benefits and risks.

Current evidence suggests that the MGB axis is a highway of communication and connections between two complex systems found in the host and the GI tract microbiota. The communication and interactions are complex and manipulating one system might also have unintentional negative outcomes for the other system. For example, high alpha diversity of the microbiota is recognized as an indicator of health. However, in early postnatal life, when the diet is primarily milk based, this diversity is low. The *Bifidobacterium* genus and *Enterobacteriaceae* family are the dominant in the microbiota at that age and these bacteria are critical to the immune development of the infant. The brain also relies on the appropriate immune development to develop.

Another possibility is to encourage the prevalence of fiber-degrading microbes in the infant microbiota by offering fiber-rich foods, but only after the infant transitions to solid foods and thereby has a sufficiently mature microbiota composition. Fiber-rich complementary foods then lead to the increased production of beneficial organic acids which impact positively the colonic epithelium where they are absorbed with specific organic acids (e.g., acetate) affection brain and cognition outcomes. The reverse is inflammatory conditions which can allow translocation of bacteria and their products to the blood where the downstream effects are more inflammation, oxidative stress and may lead to disease.

Increasing the abundance of the *Bacteroides* genus and reducing the abundance of the *Faecalibacterium genus* are counterintuitive to the beneficial effects of the *Faecalibacterium genus* on health. It is known that the methods of assessing the microbiota composition and function lack resolution to characterize which species or bacterial strains are involved. Consequently, some important changes in the microbiota composition that contribute to improve cognition in infants might be undetected in the preweaning period. Sequencing depth and resolution needs to increase to discriminate between bacterial species to better understand these relationships.

Appropriate nutrition in early postnatal life feeds the microbiota in the GI tract sets the baseline for immune, physical and brain health in later life. The microbiota shapes the immune system and is in turn shaped by the immune system. Interactions with any one of these systems impacts on the others. The ability to measure and assess such a dynamic set of systems will involve a cross disciplinary translational approach. It is possible that to reverse or at least mitigate the effects of inadequate nutrition through dietary interventions at that point in time, that narrow window of opportunity before the immune system, the brain and the microbiota mature into the stable adult shape which persists.

List of abbreviations

Full terms	Abbreviated terms
Gastrointestinal	GI
HMO	Human Milk Oligosaccharides
MAIT	Mucosal-associated invariant T cell
MHC	Major Histocompatibility Complex
Microbiota-GI-brain	MGB
NK cell	Natural Killer cell
Immunoglobulin	Ig

Author contributions

CH, NR, JM, WY, EA, MK, RD, and WM have contributed to the work. CH conceived and wrote the manuscript. JM edited and revised the manuscript and NR helped in structuring the paper and critically reviewing the manuscript. All other authors advised and critically reviewed versions of the paper. All authors approved the manuscript for publication.

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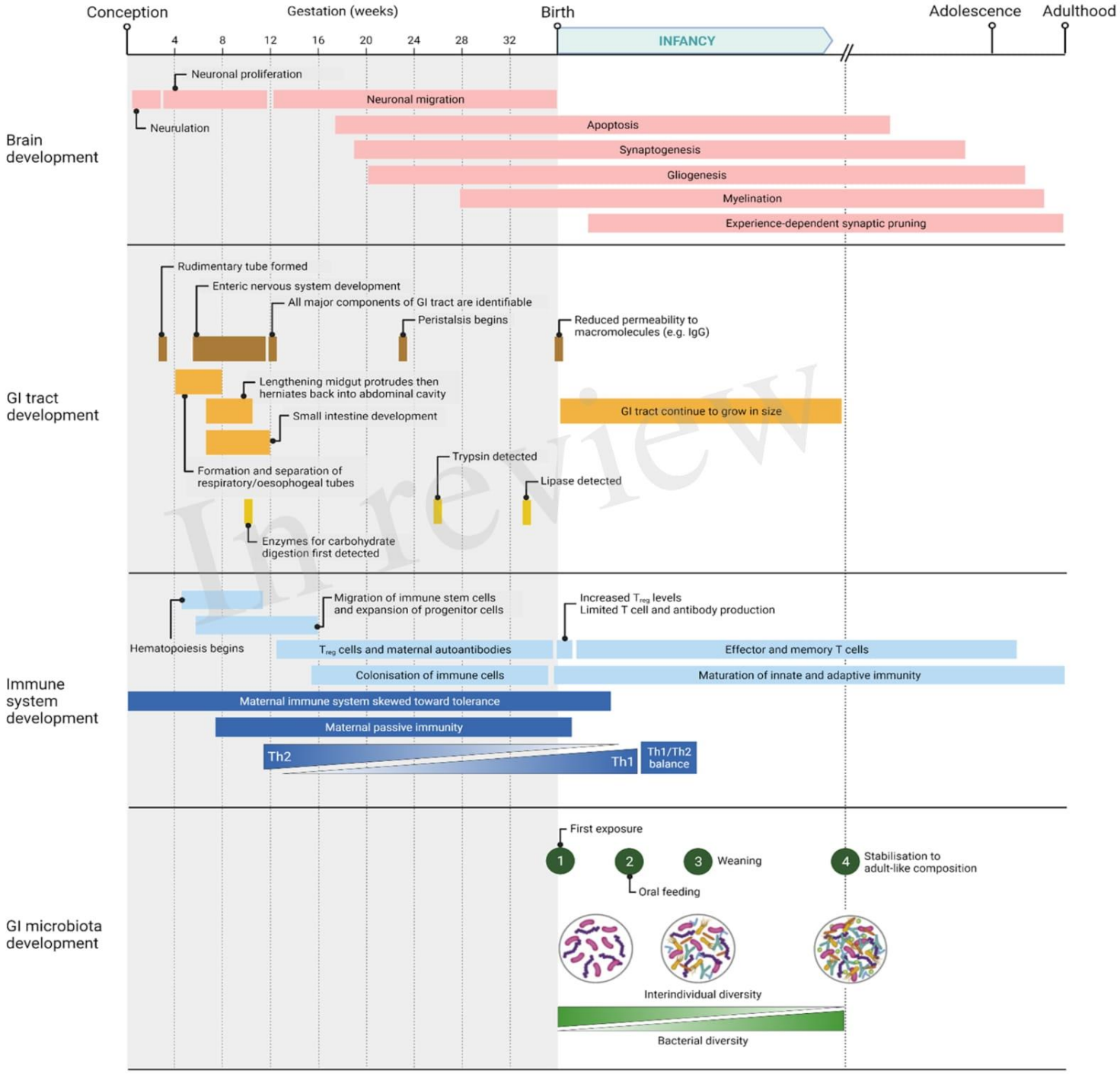
Figure 1. *Timeline of major events occurring in brain, GI tract, immune system, and GI microbiota development from conception to adulthood. Created with BioRender.com.*

Figure 1.TIF



Appendix B: Quality check report from OSS Technology Hong Kong

DNA Sample QC Sheet

Internal Order No: N2102510

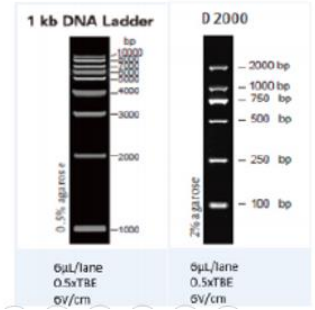
QC Reagent:	D1. Qubit® dsDNA HS Assay Kit	Lot: 2219324

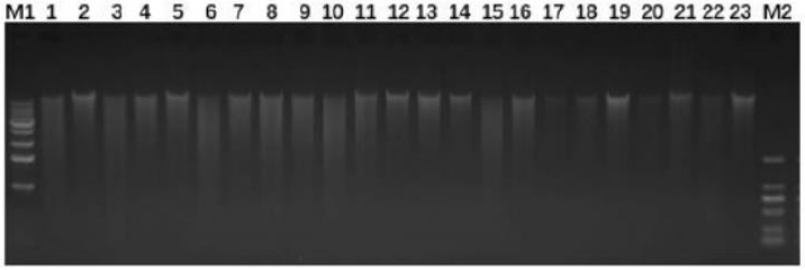
Operator: LC
QC Date: 2021-02-26

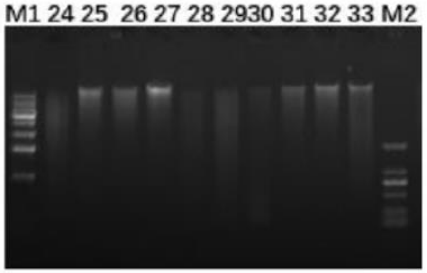
Serial No.	Customer sample ID	Internal sample ID	Internal nucleid acid ID	Sample type	volume(µl)	Nanodrop 2000				Qubit		Bioanalyzer	Rank
						N.A. conc. (ng/µl)	N.A. amount (µg)	A260/280	A260/230	N.A. conc. (ng/µl)	N.A. amount (ng)	RIN Value	
1	3PC	UDB9040	UDB9040	DNA	32	130.8	4.19	1.89	1.77	128.00	4096.00	NA	A
2	6PC	UDB9041	UDB9041	DNA	32	300.8	9.63	1.88	2.00	328.00	10496.00	NA	A
3	7PC	UDB9042	UDB9042	DNA	32	71.5	2.29	1.91	1.48	56.20	1798.40	NA	A
4	14PC	UDB9043	UDB9043	DNA	32	160.3	5.13	1.90	1.91	136.00	4352.00	NA	A
5	23PC	UDB9044	UDB9044	DNA	32	208.2	6.66	1.90	1.96	191.00	6112.00	NA	A
6	25PC	UDB9045	UDB9045	DNA	32	225.7	7.22	1.89	1.89	185.00	5920.00	NA	A
7	42PC	UDB9046	UDB9046	DNA	32	186.0	5.95	1.87	1.82	177.00	5664.00	NA	A
8	48PC	UDB9047	UDB9047	DNA	32	109.0	3.49	1.90	1.68	88.20	2822.40	NA	A
9	49PC	UDB9048	UDB9048	DNA	32	168.9	5.40	1.88	1.70	133.00	4256.00	NA	A
10	50PC	UDB9049	UDB9049	DNA	32	174.2	5.57	1.89	1.89	141.00	4512.00	NA	A
11	53PC	UDB9050	UDB9050	DNA	32	342.1	10.95	1.89	2.04	247.00	7904.00	NA	A
12	55PC	UDB9051	UDB9051	DNA	32	210.4	6.73	1.89	1.80	202.00	6464.00	NA	A
13	63PC	UDB9052	UDB9052	DNA	32	38.5	1.23	1.83	1.03	24.40	780.80	NA	A
14	64PC	UDB9053	UDB9053	DNA	28	21.9	0.61	1.78	0.51	8.90	249.20	NA	A
15	65PC	UDB9054	UDB9054	DNA	25	128.6	3.22	1.84	1.36	79.50	1987.50	NA	A
16	68PC	UDB9055	UDB9055	DNA	30	47.8	1.43	1.85	1.08	36.20	1086.00	NA	A
17	72PC	UDB9056	UDB9056	DNA	28	18.6	0.52	1.77	0.44	2.80	78.40	NA	A
18	73PC	UDB9057	UDB9057	DNA	28	16.5	0.46	1.80	0.53	4.92	137.76	NA	A
19	75PC	UDB9058	UDB9058	DNA	30	35.9	1.08	1.82	0.88	23.80	714.00	NA	A
20	76PC	UDB9059	UDB9059	DNA	28	12.2	0.34	1.96	0.48	3.96	110.88	NA	A

Serial No.	Customer sample ID	Internal sample ID	Internal nucleid acid ID	Sample type	volume(µl)	Nanodrop 2000				Qubit		Bioanalyzer	Rank
						N.A. conc. (ng/µl)	N.A. amount (µg)	A260/280	A260/230	N.A. conc. (ng/µl)	N.A. amount (ng)	RIN Value	
21	78PC	UDB9060	UDB9060	DNA	30	31.9	0.96	1.82	0.42	14.50	435.00	NA	A
22	80PC	UDB9061	UDB9061	DNA	28	17.2	0.48	1.78	0.57	5.70	159.60	NA	A
23	83PC	UDB9062	UDB9062	DNA	30	41.4	1.24	1.88	1.00	29.80	894.00	NA	A
24	TI3	UDB9063	UDB9063	DNA	28	18.3	0.51	1.73	0.46	11.80	330.40	NA	A
25	TI6	UDB9064	UDB9064	DNA	30	53.4	1.60	1.81	1.00	38.20	1146.00	NA	A
26	TI7	UDB9065	UDB9065	DNA	31	46.1	1.43	1.88	1.24	46.20	1432.20	NA	A
27	TI14	UDB9066	UDB9066	DNA	31	149.9	4.65	1.88	1.89	136.00	4216.00	NA	A
28	TI25	UDB9067	UDB9067	DNA	24	11.7	0.28	1.72	0.59	5.08	121.92	NA	A
29	TI42	UDB9068	UDB9068	DNA	24	33.9	0.81	1.83	1.00	21.60	518.40	NA	A
30	TI48	UDB9069	UDB9069	DNA	24	19.7	0.47	1.79	0.63	5.42	130.08	NA	A
31	TI50	UDB9070	UDB9070	DNA	24	14.4	0.35	1.85	0.68	9.20	220.80	NA	A
32	TI53	UDB9071	UDB9071	DNA	24	42.5	1.02	1.86	1.21	34.80	835.20	NA	A
33	TI55	UDB9072	UDB9072	DNA	24	261.1	6.27	1.87	2.08	191.00	4584.00	NA	A

Agarose gel map : Marker: 3µl Sample : sample 14,17-18,20,22,24,30-31:5ul; other sample:60ng







Appendix C: DNA sequencing report from OSS Technology Hong Kong

DNA library construction and sequencing report

I Project overview

1 Experimental workflow

Illumina high throughput DNA sequencing includes DNA extraction and quality control, library construction, quantification as well as sequencing cluster generation and high through-put sequencing. The accuracy and consistency of each step is important for the reliability of the downstream bioinformatics data analysis. We use reagents from broadly recognized source in the industry for sequencing experiment. In addition, each step is under strict quality control, and we provide detailed nucleotide quality control report as well as library quality control report. After quality control, libraries are pooled based on their effective concentration and the required sequencing data volume, which is followed by sequencing on the Illumina platform. The workflow is as follows:

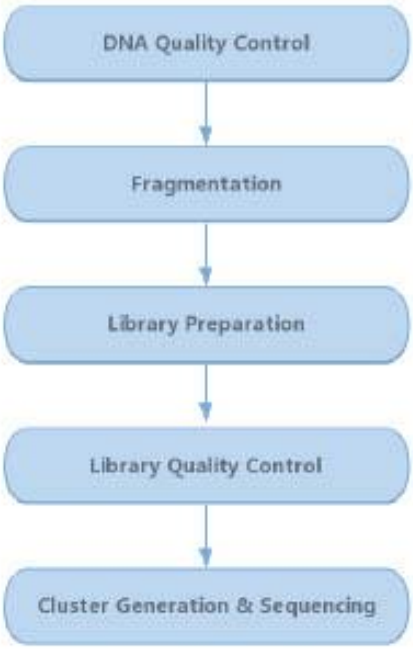


Figure 1.1 Workflow of library construction and sequencing

1.1 Library construction procedure

The starting material is DNA (100 ~ 1000ng) passed quality control. DNA is fragmented by acoustic disruption using Covaris S220 and then undergoes end repair, dA-tailing, adapter ligation and purification. The purified DNA is further selected for the right size before PCR amplification for library construction. The preliminary quantification and dilution of the library is performed using Qubit3.0, and then Agilent 2100 was used to determine the insert size and nucleic acid concentration of the resulting library sample. The effective concentration of each sample library in the library mixture is determined by qPCR before sequencing to ensure the accuracy of the sample concentration and the reliability of the sequencing data. A schematic view of library construction is shown below:

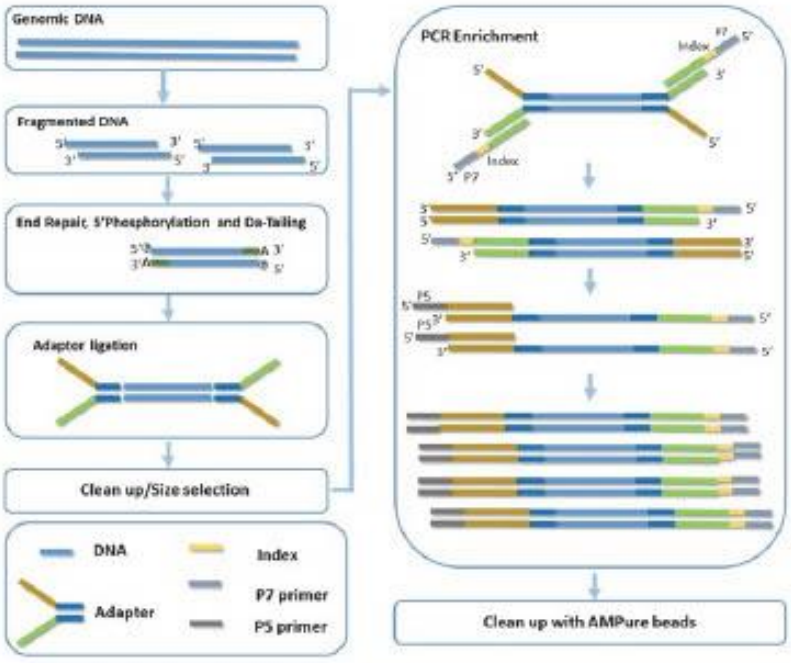


Figure 1.1.1 Schematic view of Library construction

2 Quality control workflow

Data quality assessment and filtering is a critical step in bioinformatics analysis. The quality assessment covers data volume, sequence quality distribution, base distribution, sequencing error rate, data filtering, exogenous contamination, etc.

The workflow of the quality control process from base calling to analysis result delivery is shown below:

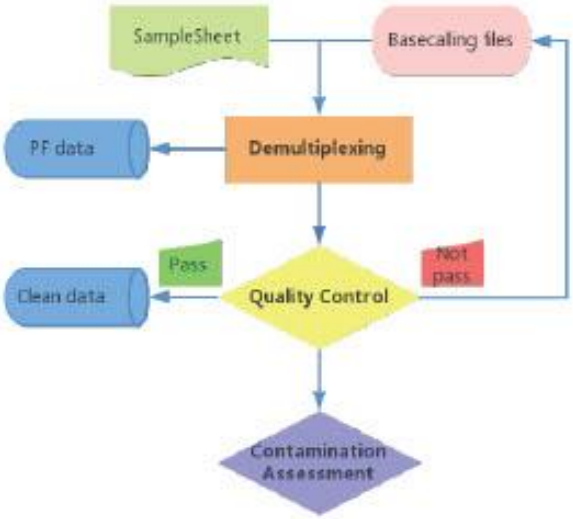


Figure 2.1 Quality control workflow

II Quality control result

1 Raw sequence data processing

Base calling was achieved with the sequencer built-in software RTA, which performs real-time conversion of the four fluorescent signals obtained from CCD to binary bcl data. Bcl data were then converted to fastq files using bcl2fastq (v2.17, part of the software package provided by Illumina). Data demultiplexing was performed simultaneously based on index information. Next, the primary analysis was performed using the sequencer build-in software HCS to determine whether the read can pass the chastity filter based on the signal quality of the first 25 cycles. If the read has no more than 2 out of the 25 cycles with chastity values below 0.6, the read is called PF (Pass Filter). PF clusters converted by Bcl2fastq are called PF data and are stored in FASTQ format.

The FASTQ format contains four lines of information for each sequence:

```
@GWZHISEQ01:289:C3Y96ACXX:6:1101:1704:2425 1:N:0:GGCTAC
GCTCTTTGCCCTTCTCGTCGAAAATTGTCTCCTCATTCGAAACTTCTCTGT
+
@@CFFFDEHHHHFIJJJ@FHGIIIEHIIJBHHHIJJEGIIJJIGH@GHCCF
```

The first and third lines contain sequence identifier information produced by the sequencer (some fastq files omit name information and leaves it empty after the "+" sign on the third line for the sake of space). The second line contains the sequence information and the fourth line contains the quality information of each corresponding base on the second line. The fourth line contains sequence quality information, and the quality score is the ASCII value of the corresponding character minus 33. For example, the ASCII value of '@'is 64, and therefore the corresponding base quality score is 31 (64-33). Starting with Illumina GA Pipeline v1.8 (currently v1.9), the range of base quality scores is from 0 to 41.

Table 1.1 Illumina sequence identifier explanation

Type	Description
GWZHISEQ01	Unique instrument name
289	Run ID
C3Y96ACXX	Flowcell ID
6	Flowcell lane
1101	Tile number within the flowcell lane
1704	'x'-coordinate of the cluster within the tile
2424	'y'-coordinate of the cluster within the tile
1	Member of a pair, 1 or 2 (paired-end or mate-pair reads only)
N	Y if the read fails filter (read is bad), N otherwise
0	0 when none of the control bits are on, otherwise it is an even number
GGCTAC	Index sequence

2 Sequence data quality assessment

RawData_QC_summary.xls
Sample_type(QM/GC/pie/).png

2.1 Sequence data quality analysis

Sequencing quality can be affected by multiple factors, including the condition of the sequencer, reagents, and sample quality. The error rate of the 5'-end bases is usually higher than average. When the sequencing cycles extend close to the 3' end, error rate rises again due to intrinsic limitations of high-throughput sequencing technology (Erlich and Mitra, 2008; Jiang et al.). The analysis on error rate distribution was used to spot bases with abnormally high error rate in the middle or toward the end of the read. The error rate of each base position on HiSeq platform should be less than 1%. The base quality scores for Illumina platforms are expressed in Q Phred, which is calculated from error rate (e) using the formula below:

formula : $Q_{phred} = -10\log_{10}(e)$

Table 2.1.1 Correlation between Illumina base calling accuracy and Qphred scores

Phred Quality Score	Probability of Incorrect Base Call	Base Call Accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10000	99.99%
50	1 in 100000	100%

The distribution of sample quality is shown below.

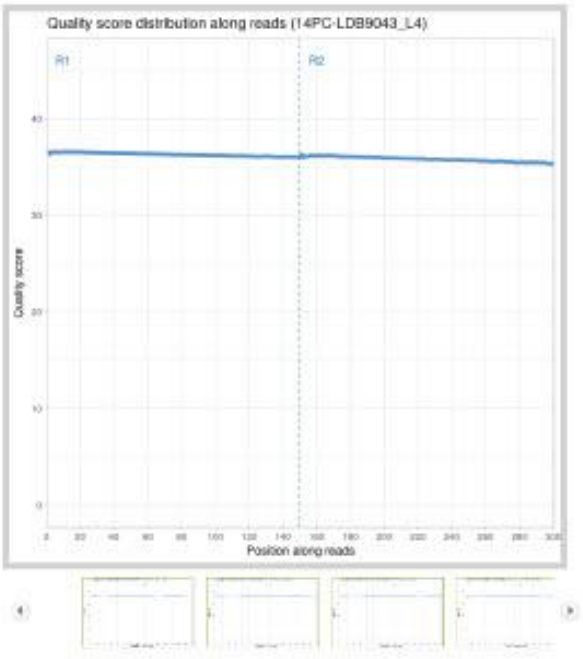


Figure 2.1.1 Data quality distribution across all bases

The X axis is the base position in the reads; the Y axis is the quality score. Q score equal to 20 indicate 1% error rate and 30 indicate 0.1%.

2.2 Base composition and distribution analysis

Base composition is analyzed to determine whether A,T,C,G were evenly distributed in each cycle and whether there is discrepancy between A/T and C/G content, which is likely to rise from the library or sequencing bias and negatively affects the data analysis. In theory, A,T,C,G percent should be more or less equal and constant in each sequencing cycle of the strand non-specific libraries based on the base complementarity of double strand DNA. In bisulfite-treated BS-seq libraries, however, unmethylated C are converted to T, resulting in high T and low C content of Read1 and high A and low G content of Read2. In terms of strand-specific sequencing, since it only covers the sequence information of one strand, the A/T or C/G content might differ for a specific sequencing cycle and display discrepancy in A/T or C/G composition. The presence of over-amplified sequences in the library in this case would further magnify the base content inconsistency of each cycle.

The base distribution of each sample is shown below:

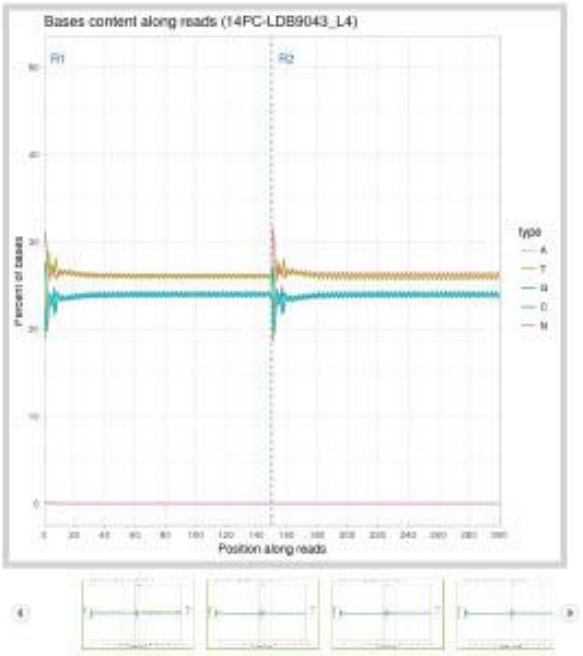


Figure 2.2.1 Base content distribution

Base content distribution. The X axis is base position of reads; the Y axis shows the percent of each of the four bases at the corresponding position. Different bases are represented by different colors as shown in the figure legend.

2.3 Sequence data quality processing

Due to the presence of short inserts and adapter dimers in the library, the sequencing data may contain adapter sequences. In addition, long fragments tend to have low

quality 3 'end bases. All these issues negatively affect the accuracy of the subsequent analysis. Therefore, the raw data were filtered based on the following criteria:

(1) remove the pair-end (PE) reads that contain the adapter sequences;
(2) remove the PE reads that the Q scores of either is below 20 for over 50% of the entire sequence;
(3) Remove the PE reads that the N composition of either is > 10%;

Illumina sequencing adapter sequence information is as follows:
P7 adapter（Read1）: AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
P5 adapter（Read2）: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

The statistics of the data filtering of each sample is summarized below.

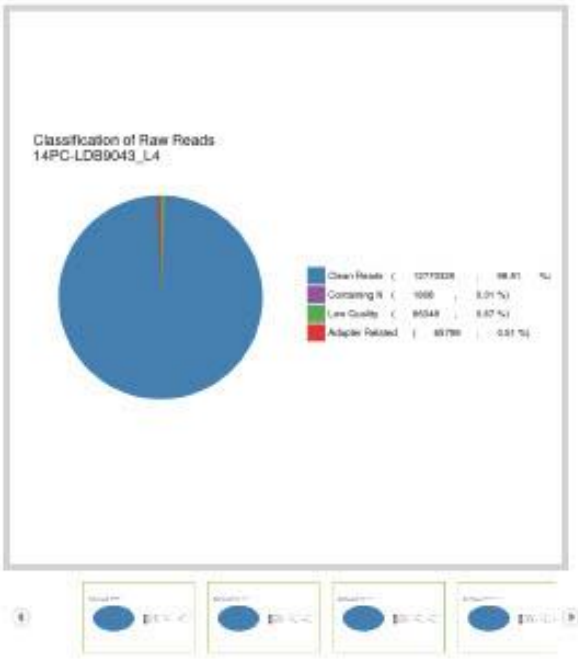


Figure 2.3.1 Read classification and statistics based on raw reads quality processing

Clean Reads: the number and percent of clean reads obtained after filtering; Containing N: the number and percent of reads with over 10% of undetermined bases (N); Low Quality: the number and percent of reads filtered out due to low quality; Adapter Related: the number and percent of reads that were filtered out due to the detection of the adapter sequences.

2.4 Raw data statistics

The volume of the raw and filtered data as well as the quality assessment are summarized in the table below.

Table 2.4.1 PF data statistics

Library	Sample	Raw Reads	Raw Base(G)	Error Rate(%)	Q20(%)	Q30(%)	GC Content(%)
14PC-LDB9043_L4	14PC-LDB9043	12924281	3.877	0.04	97.90	93.89	47.66
23PC-LDB9044_L4	23PC-LDB9044	13236908	3.971	0.04	97.88	93.89	49.25
25PC-LDB9045_L4	25PC-LDB9045	13059425	3.918	0.04	98.06	94.41	49.16
3PC-LDB9040_L4	3PC-LDB9040	12089783	3.627	0.04	98.00	94.28	49.22
42PC-LDB9046_L4	42PC-LDB9046	15040721	4.512	0.04	97.92	93.93	46.45
48PC-LDB9047_L4	48PC-LDB9047	12908445	3.873	0.04	97.71	93.44	47.17
49PC-LDB9048_L4	49PC-LDB9048	12047210	3.614	0.04	97.97	94.15	48.44
50PC-LDB9049_L4	50PC-LDB9049	14690337	4.407	0.04	97.87	93.91	49.43
53PC-LDB9050_L4	53PC-LDB9050	15735409	4.721	0.04	97.65	93.63	47.32
55PC-LDB9051_L4	55PC-LDB9051	13416523	4.025	0.04	97.85	93.88	50.38
63PC-LDB9052_L4	63PC-LDB9052	15484957	4.645	0.04	97.84	93.82	49.81
64PC-LDB9053_L4	64PC-LDB9053	13307287	3.992	0.04	97.86	94.00	50.91
65PC-LDB9054_L4	65PC-LDB9054	11574977	3.472	0.04	97.45	93.10	46.08
68PC-LDB9055_L4	68PC-LDB9055	13632572	4.090	0.04	97.83	93.75	47.44
6PC-LDB9041_L4	6PC-LDB9041	12356600	3.707	0.04	97.48	93.24	46.43
72PC-LDB9056_L4	72PC-LDB9056	14095840	4.229	0.04	97.81	93.69	45.73
73PC-LDB9057_L4	73PC-LDB9057	12088613	3.627	0.04	97.78	93.74	48.47
75PC-LDB9058_L4	75PC-LDB9058	13042216	3.913	0.04	97.95	94.16	48.38
76PC-LDB9059_L4	76PC-LDB9059	11648217	3.494	0.04	97.95	94.18	48.89
78PC-LDB9060_L4	78PC-LDB9060	12040883	3.612	0.04	98.03	94.07	42.70
7PC-LDB9042_L4	7PC-LDB9042	12093061	3.628	0.04	98.09	94.38	48.20
80PC-LDB9061_L4	80PC-LDB9061	11020924	3.306	0.04	97.78	93.69	46.34
83PC-LDB9062_L4	83PC-LDB9062	15218452	4.566	0.04	97.47	92.98	49.55
TI14-LDB9066_L4	TI14-LDB9066	11182973	3.355	0.04	96.59	91.70	45.48
TI25-LDB9067_L4	TI25-LDB9067	11821569	3.546	0.04	97.06	92.57	44.30
TI3-LDB9063_L4	TI3-LDB9063	14500390	4.350	0.04	96.81	91.97	43.58
TI42-LDB9068_L4	TI42-LDB9068	11409355	3.423	0.04	97.06	92.52	43.00
TI48-LDB9069_L4	TI48-LDB9069	12061878	3.625	0.04	97.82	94.03	44.35
TI50-LDB9070_L4	TI50-LDB9070	12752969	3.826	0.04	97.11	92.82	44.47
TI53-LDB9071_L4	TI53-LDB9071	11690193	3.507	0.04	97.02	92.44	43.16
TI55-LDB9072_L4	TI55-LDB9072	14278330	4.283	0.04	96.59	91.50	43.58
TI6-LDB9064_L4	TI6-LDB9064	12429994	3.729	0.04	96.89	92.22	44.47
TI7-LDB9065_L4	TI7-LDB9065	12137912	3.641	0.04	97.31	93.04	44.54

Description :

(1) Library: Library ID.
(2) Sample: Sample ID.
(3) Raw Reads: The total read count of the raw data; the number is the number of lines in the raw fastq files divided by 4.
(4) Raw Bases（G）: The total base count in the raw sequence.
(5) Q20：The percentage of bases with Q scores higher than 20.
(6) Q30：The percentage of bases with Q scores higher than 30.
(7) GC Content：The GC percent in the sequence.

III References

[1] Cock, P.J.A., Fields, C.J., Goto, N., Heuer, M.L., and Rice, P.M. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic acids research 38, 1767-1771.
[2] Erlich, Y., and Mitra, P.P. (2008). Alta-Cyclic: a self-optimizing base caller for next-generation sequencing. Nature methods 5, 679-682.
[3] Jiang, L., Schlesinger, F., Davis, C.A., Zhang, Y., Li, R., Salit, M., Gingeras, T.R., and Oliver, B. Synthetic spike-in standards for RNA-seq experiments. Genome research 21, 1543-1551.
[4] Hansen, K.D., Brenner, S.E., and Dudoit, S. Biases in Illumina transcriptome sequencing caused by random hexamer priming. Nucleic acids research 38, e131-e131.

IV Appendix

1 File directory

2 Notes

We suggest the result files be opened with a professional text editor such as Excel or EditPlus.

When opening a report using Internet Explorer, if it returns "for security reasons, Internet Explorer has restricted this page from running scripts or ActiveX Controls that can access your computer. Click here for options ..." Please select 'Allow' to view the report.

Appendix D: Sequenced data quality

D.1. Taxonomical composition

Sample	Raw		Trimmed and host removed		Kingdom and domain assignment counts				
	Number of read pairs	bp	Number of read pairs	bp	Bacteria	Archaea	Fungi	Eukaryota - non fungi	Viruses
14PC-LDB9043	12924281	3877284300	12101733	3382664301	10086025	45004	0	11073	0
23PC-LDB9044	13236908	3971072400	12749974	3549306074	11162154	25427	0	17112	52870
25PC-LDB9045	13059425	3917827500	12416297	3428722064	10786451	55991	0	28177	43653
3PC-LDB9040	12089783	3626934900	11363219	3151819935	10210086	30951	0	6180	73844
42PC-LDB9046	15040721	4512216300	13916756	3862413371	10547499	54484	0	8249	1326171
48PC-LDB9047	12908445	3872533500	11787880	3295626671	10186564	33905	0	12608	182372
49PC-LDB9048	12047210	3614163000	11274134	3139707232	9384816	98684	0	39016	7326
50PC-LDB9049	14690337	4407101100	13616478	3787711026	11761297	101530	0	15641	61698
53PC-LDB9050	15735409	4720622700	10041638	2775831356	7388821	62310	0	54627	0
55PC-LDB9051	13416523	4024956900	13009063	3652420024	10538161	80280	0	13724	0
63PC-LDB9052	15484957	4645487100	15020411	4175908568	12752464	79373	0	20899	0
64PC-LDB9053	13307287	3992186100	12373666	3443266657	9872204	111550	0	17197	385266

65PC-LDB9054	11574977	3472493100	7706024	2139540772	6791337	54469	0	0	36335
68PC-LDB9055	13632572	4089771600	12940504	3614729977	11626242	26564	0	15864	97022
6PC-LDB9041	12356600	3706980000	5508327	1529976528	4871510	9299	0	8297	10989
72PC-LDB9056	14095840	4228752000	13082513	3598607286	11258456	49237	0	12394	8373
73PC-LDB9057	12088613	3626583900	11406455	3160285882	9823000	56076	0	37309	0
75PC-LDB9058	13042216	3912664800	12252149	3429855761	10564388	25744	0	28296	218426
76PC-LDB9059	11648217	3494465100	10939925	3028241919	8438925	91641	0	18093	177412
78PC-LDB9060	12040883	3612264900	11733792	3250815099	6909573	25985	0	6943	2242651
7PC-LDB9042	12093061	3627918300	11315071	3119876298	9449407	54708	0	26151	83819
80PC-LDB9061	11020924	3306277200	10804050	2972199867	8171629	40053	0	12109	483099
83PC-LDB9062	15218452	4565535600	14808565	4128660935	12451746	22528	0	44286	176462
TI14-LDB9066	11182973	3354891900	309854	83956936	48688	67	0	55725	599
TI25-LDB9067	11821569	3546470700	1683701	465282306	1571097	0	0	1825	1234
TI3-LDB9063	14500390	4350117000	385646	105175806	314601	0	0	1369	5818
TI42-LDB9068	11409355	3422806500	1027826	280651926	219395	0	0	1827	559227
TI48-LDB9069	12081878	3624563400	7052729	1896858079	6124166	0	0	14062	532944

TI50-LDB9070	12752969	3825890700	218511	56233683	134743	0	2768	21728	967
TI53-LDB9071	11690193	3507057900	716375	197292299	638775	0	1805	822	1794
TI55-LDB9072	14278330	4283499000	134435	35569575	55345	0	0	12366	304
TI6-LDB9064	12429994	3728998200	1007947	278362335	923216	0	0	6817	3834
TI7-LDB9065	12137912	3641373600	3303548	919733750	3179119	0	0	1777	0

D.2. KEGG and SEED

Sample	Raw		Trimmed and host removed		Kingdom and domain assignment counts						
	Number of read pairs	bp	Number of read pairs	bp	Bacteria	Archaea	Fungi	Eukaryota - non fungi	Viruses	Assigned KEGG counts	Assigned SEED counts
14PC-LDB9043	12924281	3877284300	12101733	3382664301	10086025	45004	0	11073	0	3479577	3623504
23PC-LDB9044	13236908	3971072400	12749974	3549306074	11162154	25427	0	17112	52870	2622756	3955008
25PC-LDB9045	13059425	3917827500	12416297	3428722064	10786451	55991	0	28177	43653	3428811	3534017
3PC-LDB9040	12089783	3626934900	11363219	3151819935	10210086	30951	0	6180	73844	2608010	3606114
42PC-LDB9046	15040721	4512216300	13916756	3862413371	10547499	54484	0	8249	1326171	3426367	3797512
48PC-LDB9047	12908445	3872533500	11787880	3295626671	10186564	33905	0	12608	182372	3305654	3364378

49PC-LDB9048	12047210	3614163000	11274134	3139707232	9384816	98684	0	39016	7326	2665294	3205299
50PC-LDB9049	14690337	4407101100	13616478	3787711026	11761297	101530	0	15641	61698	3173244	3879621
53PC-LDB9050	15735409	4720622700	10041638	2775831356	7388821	62310	0	54627	0	2166936	2796005
55PC-LDB9051	13416523	4024956900	13009063	3652420024	10538161	80280	0	13724	0	3286979	3830774
63PC-LDB9052	15484957	4645487100	15020411	4175908568	12752464	79373	0	20899	0	3913134	4389787
64PC-LDB9053	13307287	3992186100	12373666	3443266657	9872204	111550	0	17197	385266	3352934	3575620
65PC-LDB9054	11574977	3472493100	7706024	2139540772	6791337	54469	0	0	36335	2097169	2240772
68PC-LDB9055	13632572	4089771600	12940504	3614729977	11626242	26564	0	15864	97022	3626981	3883065
6PC-LDB9041	12356600	3706980000	5508327	1529976528	4871510	9299	0	8297	10989	1098116	1799126
72PC-LDB9056	14095840	4228752000	13082513	3598607286	11258456	49237	0	12394	8373	3775458	3465289
73PC-LDB9057	12088613	3626583900	11406455	3160285882	9823000	56076	0	37309	0	2875273	3280038
75PC-LDB9058	13042216	3912664800	12252149	3429855761	10564388	25744	0	28296	218426	3483542	3490363
76PC-LDB9059	11648217	3494465100	10939925	3028241919	8438925	91641	0	18093	177412	2428432	2885509
78PC-LDB9060	12040883	3612264900	11733792	3250815099	6909573	25985	0	6943	2242651	2035133	2109967
7PC-LDB9042	12093061	3627918300	11315071	3119876298	9449407	54708	0	26151	83819	2648364	3414641
80PC-LDB9061	11020924	3306277200	10804050	2972199867	8171629	40053	0	12109	483099	2257735	2540527

83PC-LDB9062	15218452	4565535600	14808565	4128660935	12451746	22528	0	44286	176462	3568100	4392271
TI14-LDB9066	11182973	3354891900	309854	83956936	48688	67	0	55725	599	7819	6496
TI25-LDB9067	11821569	3546470700	1683701	465282306	1571097	0	0	1825	1234	443309	685898
TI3-LDB9063	14500390	4350117000	385646	105175806	314601	0	0	1369	5818	155686	128846
TI42-LDB9068	11409355	3422806500	1027826	280651926	219395	0	0	1827	559227	40141	115194
TI48-LDB9069	12081878	3624563400	7052729	1896858079	6124166	0	0	14062	532944	2465514	2609550
TI50-LDB9070	12752969	3825890700	218511	56233683	134743	0	2768	21728	967	53405	43859
TI53-LDB9071	11690193	3507057900	716375	197292299	638775	0	1805	822	1794	400767	288825
TI55-LDB9072	14278330	4283499000	134435	35569575	55345	0	0	12366	304	11975	13260
TI6-LDB9064	12429994	3728998200	1007947	278362335	923216	0	0	6817	3834	81914	402207
TI7-LDB9065	12137912	3641373600	3303548	919733750	3179119	0	0	1777	0	631575	1434722