Effects of dietary caprine milk oligosaccharides enriched fraction on maternal large intestine and the consequences for the development of the offspring

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Abstract

The colonisation of the neonate gastrointestinal tract by health-promoting microbiota is likely to improve the overall health of the infant and may also have health benefits in later life. Initial development and maturation of the foetal/neonatal gastrointestinal tract is heavily influenced by the *in utero* environment which itself, may be altered by the maternal diet and gastrointestinal tract microbiota composition. The maternal gastrointestinal tract microbiota can be altered by supplementation with synthetic oligosaccharides; however, positive effects on the health and well-being of the offspring have not been adequately established. Human milk contains natural oligosaccharides known to improve the gastrointestinal tract colonisation and the development and maturation of the infant gastrointestinal tract. Among domestic farm animals, caprine milk has oligosaccharides structurally similar to human milk and potentially similar beneficial effects for the infant. We hypothesised that feeding caprine milk oligosaccharide enriched product to pregnant and lactating mice would induce changes in the maternal large intestine microbiota and milk composition, accelerating the development and maturation of the offspring's large intestine tissue and altering the gastrointestinal tract microbiota composition. The aim of this project was to obtain bifidobacteria from the faeces of breast-fed human infants and determine which were of capable fermenting caprine milk oligosaccharide enriched product. Subsequently, the effects of the best strains on the morphology and metabolic pathways of the colonic mucosa of germ-free and conventionally raised mice, supplemented with dietary caprine milk oligosaccharide enriched product.

The present study is the first to report New Zealand Saanen caprine colostrum, milk and whey. An enrichment method previously described was used to produce a caprine milk oligosaccharide enriched product for *in vitro* and *in vivo* assessment of its health effects. Caprine milk oligosaccharide enriched product was shown to differentially stimulate the growth of bifidobacteria, commonly found in the gastrointestinal tract of breast-fed infants.

Among the bifidobacterial species tested, *Bifidobacterium bifidum* utilised caprine milk oligosaccharide enriched product most efficiently when compared to *Bifidobacterium breve* and *Bifidobacterium longum* subsp*. longum. B. bifidum* (AGR2166) was shown to ferment the sialyloligosaccharides, 3'- and 6'-sialyl-lactose present in caprine milk oligosaccharide enriched product through cell-associated sialidase expression. Augmented microbial biomass associated with enhanced growth and *in vitro* fermentation of caprine milk oligosaccharide enriched product, increased the production of microbial fermentation end products such as acetate and lactate. These findings indicate that *in vivo* caprine milk oligosaccharide enriched product may stimulate the growth and fermentation of bifidobacteria within the gastrointestinal tract.

Germ-free mice or mice mono-associated with *B. bifidum* (AGR2166) were used to test the *in vivo* effects of maternal caprine milk oligosaccharide enriched product consumption during pregnancy and the effects on the foetus. Caprine milk oligosaccharide enriched product diet showed no effects on maternal gastrointestinal tract or foetal growth regardless of microbial status. Mice inoculated with *B. bifidum* (AGR2166) and fed caprine milk oligosaccharide enriched product diet, however, showed an increased bacterial translocation from maternal gastrointestinal tract to organs and placenta (inferred by the presence of the bifidobacteria 16S rRNA gene in the maternal organs). Increased translocation of commensal bacteria from maternal gastrointestinal tract to the foetus may have important effects on foetal immunological programming.

The consumption of caprine milk oligosaccharide enriched product, during gestation and lactation were also tested in conventional rodents and it had no effects on maternal gastrointestinal tract microbiota and morphology. Changes on maternal lipid metabolism and increased maternal milk protein, however, were observed. These modifications may have positively affected the development of the pups, relative abundance of gastrointestinal tract bifidobacteria and butyric acid production at weaning. Important changes in the plasma and

urine metabolites involved in bile acid and fatty acid metabolism were also observed in the pups as a consequence of maternal caprine milk oligosaccharide-enriched diet. The effects of maternal caprine milk oligosaccharide enriched product diet on pups, were no longer apparent after 30 days of consuming a control diet post-weaning, however, detrimental physiological characteristics such as an increased body fat were observed. Further studies, are needed to understand the physiological effects of caprine milk oligosaccharides on the maternal/infant pair.

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Table of contents

List of figures

List of tables

List of abbreviations

afu	Absolute fluorescent units
ANOVA	Analysis of variance
AOAC	Association of official analytical chemistry
ARDRA	Amplified ribosomal DNA restriction analysis
BMI	Body mass index
BMO	Bovine milk oligosaccharides
CD4+ T cells	T helper cells expressing the surface protein CD4
cfu	Colony forming units
CMO	Caprine milk oligosaccharide
CMOP	Caprine milk oligosaccharide enriched product
CNS	Central nervous system
CpG	Cytosine phosphate guanine
CRM	Certified reference material
CRAMP	Cathelicidin-related antimicrobial peptide
DC	Dendritic cells
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
ENS	Enteric nervous system
FDR	False discovery rate
FL	Fucosyllactose
FOS	Fructo-oligosaccharides
Fuc	Fucose
FUT	Fucosyltransferase
GAL	Galactose
GALT	Gut-associated lymphoid tissue

GC-FID	Flame ionisation detector gas chromatography
GF	Germ-free
GHS	General health score
GIT	Gastrointestinal tract
GLC	Glucose
GLCNac	N-acetyl-glucosamine
GOD	Glucose oxidase
GOS	Galactooligosaccharides
Hib	*Haemophilus influenzae*
HILIC	Hydrophilic interaction liquid chromatography
HLA	Human leukocyte antigen
HMO	Human milk oligosaccharides
HPLC	High performance liquid chromatography
TICP-OS	Inductively coupled plasma atomic emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectroscopy
IECs	Intestinal epithelial cells
IELs	Intraepithelial lymphocites
Ig	Immunoglobulin
IκB	Nuclear factor kappa inhibitor
IL	Interleukin
ILFs	Isolated lymphoid follicles
INFγ	Interferon gamma
LacNac	N-acetyllactosamine
LC-MS	Liquid chromatography-mass spectrometry
LDLs	Lamina propria lymphocytes
Le	Lewis group
LNB	Lacto-N-biose

LPLs	Lamina propria lymphocytes
LPS	Lipopolysaccharide
LSD	Least significant difference
LTi	Lymphoid tissue inducer
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MTPY	Modified TPY agar
m/z	Mass to charge ratio
NCC	Neural crest cells
NeuAc	N-acetylneuraminic acid
NF-κB	Nuclear factor Kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
OD	Optical density
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
POD	Catalysis of peroxidase
PUFA	Polyunsaturated fatty acids
RISA	Ribosomal intergenic spacer analysis
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCFA	Short chain fatty acids
SI	Small intestine
SL	Sialyllactose
spp.	Species

TGF	Transforming growth factor beta
Th	Cellular T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TReg	Lymphocyte regulatory T cell
TTGE	Temperature gradient gel electrophoresis
VIP	Variable importance in projection
4Mu-Neu5Ac	4-methylubelliferyl-a-D-*N*-acetylneuraminic acid

Chapter 1 General Introduction

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Chapter 1

1.1 Introduction

There has been a shift in the cause of mortality in the last two centuries. Until the early part of the 20th century, the predominant causes of death were either infectious diseases (including tuberculosis and malaria), or war-related [1]. Today, non-communicable diseases such as cancer and metabolic diseases (insulin resistance type 2 diabetes, obesity, hypertension, and cardiovascular diseases) are the biggest contributors to human morbidity and mortality [2, 3]. This shift in the cause of mortality has been driven largely by improved sanitation, cleaner water supply and the availability of antimicrobial agents [4, 5], combined with a simultaneous increase in energy intake and reduction in physical activity [6]. It is well established that lifestyle factors (such as tobacco smoking, obesity, sedentary lifestyle, and dietary excess or imbalance) and genetics play a major role in the development of adult metabolic diseases [7, 8]. Over the last 20 years, however, a growing body of evidence supports the hypothesis that insults during critical periods of development may predispose the organism for later diseases by “programming” or altering organ structure and function [9-11]. This process has been termed the Developmental Origins of Health and Disease, or Developmental Programming [12-16].

In the late 1980s, the quality and quantity of maternal dietary intake during pregnancy and lactation was identified as one of the most influential, but modifiable, factors that promote altered foetal growth and organ development [17-19]. As a result a large number of studies on foetal over- and under-nourishment were conducted [1, 3–5]. Those studies outlined the plasticity existing during development, which allows a range of different phenotypes to emerge from a single genotype, largely in response to the nature of the environmental cues received during pregnancy [20]. This environmental modulation, occuring during critical windows of development, has important evolutionary implications [20]. A mismatch between the environment predicted during the perinatal period and the actual environment

encountered later in life, for example, is suggested to be causal in the increased susceptibility to obesity, leptin and insulin resistance, elevated blood pressure and cardiovascular dysfunction in the progeny [21, 22]. Most organs including adipose tissue, pancreas, kidney, skeletal muscle, brain and gastrointestinal tract (GIT) have been reported to be imprinted by early disturbances [23].

Maternal nutrition also became the focus of many studies trying to improve the perinatal environment and thereby foetal/neonatal health. Studies with iron and folic acid supplementation of the mother's diet, for example, showed measurable benefits to the offspring such as decreased incidences of neural tube defects and decreased chances of early neonatal death [24, 25]. More recently, probiotic and prebiotic supplements that influence maternal GIT microbiota composition and function have been reported to beneficially impact the development and maturation of the neonatal GIT [26, 27]. An improved maternal microbiota is likely to provide beneficial microbes for direct colonisation of the neonatal GIT, and affecting the succession of commensal microbiota.

Interactions between the microbiota and the host are potential players in the early programming of GIT function and host organ metabolism [28-30]. In humans, early alterations in GIT microbial colonisation, for example, by perinatal antibiotic treatment, are suspected to be responsible for increased disease risks in later life [30, 31]. Consequently, the transition between pre- and postnatal life is likely to be a critical window for maternal dietary intervention to ensure normal development of the infant GIT, with potential long term effects on GIT and other organ health.

1.2 Gastrointestinal tract development and maturation

The GIT has a major role in health. It is where food and drink are digested and nutrients absorbed to support the body's growth, maintenance and repair mechanisms. The lower GIT (small and large intestine) contain a large number of microorganisms in symbiosis with the

host, and it is also a prominent part of the immune system. GIT development and maturation starts soon after conception, and extends through neonatal life, with further modifications occurring during the transition from milk to solid food at weaning [32]. The GIT undergoes tremendous growth during foetal life. It elongates 1000 fold from 5 to 40 weeks. The length doubles in the last 15 weeks of gestation, reaching a mean length at birth of 275 cm [32]. The GIT epithelium is the first to differentiate and specialise. In the small intestine (SI), for example, the villi (small finger like projections into the intestinal lumen), are already formed at 16 weeks of gestation. In the large intestine, villi are also present, but these partially regress at around 29 weeks of gestation [33]. Microvilli begin to cover the apical surface of the small intestinal epithelium so that by adulthood, this surface provides the largest interface between the outside environment and the internal milieu (approximately 200 m^2) [32].

1.2.1 Epithelium

The epithelial absorptive lining of the primitive GIT is initially stratified but becomes a single layer of columnar cells at the end of the first gestational trimester [33]. The epithelial enterocyte derives from the same undifferentiated stem cell that gives rise to columnar absorptive epithelium (enterocytes), mucus producing goblet cells (involved in the secretion of the mucous lining), Paneth cells (involved in secretion of defensins and other peptides implicated in the innate immunity), and M cells (specialised for delivering foreign antigens and microorganisms to organised lymphoid tissues within the mucosa of the small and large intestines; Figure 1.1), and other cell types involved in the local neuroendocrine and immune systems [33, 34]. The function of these cells beyond nutrient absorption is well recognised and some of their major roles include bacterial mucosal cross talk and innate barrier function [35].

As the stratified epithelium is converted to a single layer, polarisation of the enterocytes is established, forming distinct apical, lateral, and basal cell surfaces, critical to the functional

differentiation of the epithelium. Apical junctional complexes inhibit free diffusion of membrane proteins and separate different functional compartments. The apical membrane carries digestive hydrolases and transporters, the lateral membrane carries mostly transporters, and the basal membrane carries receptors, such as the integrins, for basement membrane components [33, 36, 37]. The adherens junctions contain E-cadherin, which both binds adjacent cells and interacts with intracellular components such as catenins, forming links to the cytoskeleton. Intermediate filaments, or cytokeratins, form a significant portion of the cytoskeleton [33].

Epithelial maturation continues during late gestation and infancy. This process is modulated by various cytokines and growth factors present in the systemic circulation, interstitial fluid, and ingested amniotic fluid and human milk [33]. Mucosal growth involves fission and deepening of crypts, increases in villus width and number, and appearance of submucosal folds. A second phase of epithelial hyperplasia is observed at weaning [38] (Figure 1.1).

1.2.2 Neural system

The enteric nervous system (ENS) is a large network of neurons and glial cells located along the length of the GIT. It has been estimated that there are more neurons within the GIT than there are in the spinal cord [39]. Most of the neurons are located in either myenteric ganglia, which are situated between the longitudinal and circular muscle layers, or in submucosal ganglia within the submucosal connective tissue. The ENS autonomously controls or regulates many functions, including motility, secretion, vascular tone, and hormone release, although the central nervous system (CNS) can modulate these behaviours [40]. It normally connects with the CNS through the parasympathetic and sympathetic nervous systems. In general, sympathetic stimulation causes inhibition of GIT secretion and motor activity, and contraction of GIT sphincters and blood vessels. Conversely, parasympathetic stimuli typically stimulate these digestives activities [41]. Through these connections, the GIT can

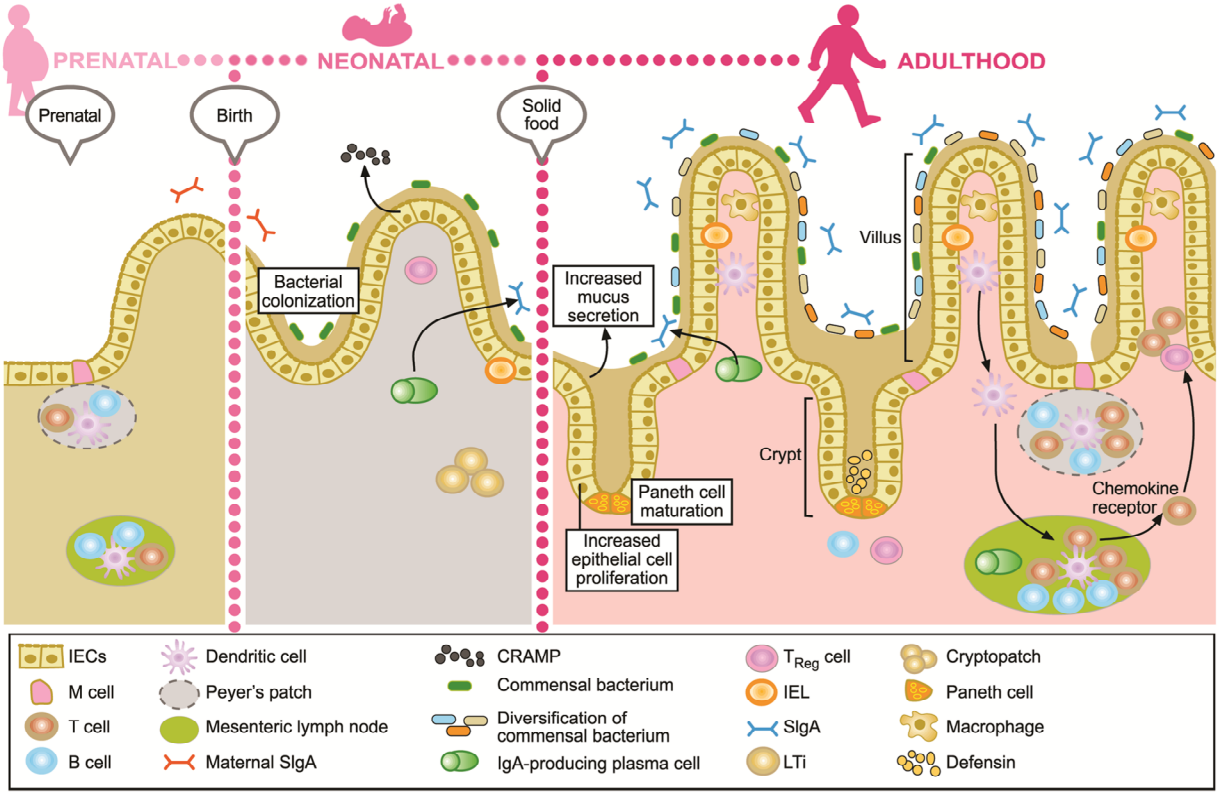


Figure 1.1 Postnatal development and maturation of the gastrointestinal tract mucosal barrier and immune system. Both developmental and environmental signals drive significant changes of the intestinal epithelium during the postnatal period and accompany the establishment of an increasingly complex and dense gastrointestinal tract microbiota. The neonatal intestinal mucosa is characterised by low levels of epithelial cell proliferation, the absence of crypts and crypt-based Paneth cells, and expression of cathelicidin-related antimicrobial peptide (CRAMP); by contrast, the formation of intestinal crypts late during the second week after birth initiates increased proliferation and rapid epithelial cell renewal and the generation of α-defensin-producing Paneth cells. A decrease in the level of expression of Toll-like receptor 4 (TLR4) by epithelial cells before birth, and a steady increase in the intestinal expression level of the nuclear NF-κB inhibitor (IκB) during the postnatal period decrease the responsiveness to bacterial lipopolysaccharide and other pro-inflammatory stimuli. Simultaneously, the acquisition of epithelial TLR tolerance creates a neonatal period of decreased innate immune responsiveness. The secondary lymphoid structures of Peyer's

patches and lymph nodes are generated before birth in mice and humans, and these mature during the postnatal period. By contrast, cryptopatches and isolated lymphoid follicles (IFLs) are formed after birth in mice. Specialised epithelial cells, known as M cells, reside above ILFs and Peyer's patches and facilitate antigen transport from the lumen to the underlying lymphoid cells. Simultaneously, innate lymphocytes (such as lymphoid tissue inducer (LTi) cells) and T cells leave the liver and thymus, respectively, and colonise the enteric mucosal tissue, including the epithelium. Intraepithelial lymphocytes (IELs) reside in close proximity to the epithelium. Also, increasing numbers of dendritic cells and macrophages migrate to the intestinal mucosa. In contrast to innate lymphocytes, regulatory T (TReg) cells populate the intestinal mucosa in response to bacterial colonisation. Although B cells are present in intestinal tissue during early development, plasma cells producing dimeric immunoglobulin A (IgA) are only generated after birth to provide secretory IgA (SIgA) to the lumen. Maternal SIgA is provided by breast milk during the early postnatal period. Figure adapted from Renz *et al.*, 2012, [42].

provide sensory information to the CNS affecting GIT function [43]. Connection to the CNS also means that signals from outside of the GIT can be transmitted to the digestive system: for instance, the sight of appealing food stimulates secretion in the stomach.

The ENS is entirely derived from neural crest cells (NCC), a transient population of multipotent cells that arises between the dorsal neural tube and the overlying ectoderm during neurulation in the early embryo. NCC migrate extensively along defined pathways throughout the embryo and give rise to a diverse range of cell types, including neuronal and glial cells of the peripheral nervous system and autonomic nervous system, mesenchymal cells of the head (eg, cartilage, bone, connective tissue, smooth muscles), chromaffin cells of the adrenal gland, and pigment cells of the skin [44]. To form a mature, functional ENS, NCC must not only migrate along the entire length of the GIT, but also proliferate extensively, differentiate into a wide range of phenotypic classes of neurons, and become functionally active. In the embryonic human GIT, the migrating NCC reach the mid intestine by week 5 of gestation, and the entire length is colonised by week 7. NCC derives precursors along the length to form the myenteric plexus, providing motor innervation to both layers of the muscle required later for motility [45].

The coordinated neuromuscular activity of the GIT requires the interaction of enteric neurons, smooth muscle, and interstitial cells of Cajal (ICC) [46, 47]. A well-defined band of circular muscle is apparent within the esophagus at week 8 of gestation and in the large intestine at week 11. By week 14, the smooth muscle layers of the large intestine are well defined with concentric muscularis mucosae, circular muscle, and longitudinal muscle apparent [45]. ICC (which, like the smooth muscle, are derived from mesoderm), and its development generates the autonomic intestinal motility [48]. From weeks 12 to 14, ICC become discrete and tightly distributed around the myenteric ganglia along the entire GIT. By week 14, the human GIT has a relatively mature appearance due to the presence of

concentric muscle layers, submucosal and myenteric plexuses, and ICC networks that are associated with the ENS [45].

Mammals swallow amniotic fluid, and the intestinal contents (meconium) needs to be propagated anally. The types of motility patterns, and the mechanisms controlling motility during foetal development have been most extensively studied in zebra fish and mice. Organised and spontaneous contractions that propagate both orally and anally are observed in mice within 2 to 10 days before birth, depending on the GIT segment studied [49]. In humans, however, studies are limited. Studies in which radio-opaque molecules were introduced into the amniotic fluid of 37- to 40-gestational-week pregnant women, have shown that the dye is present in the foetal colon between 4.5 to 7 hours after its introduction [50]. At birth, GIT motility patterns were reported to be immature compared to those seen in adults [51, 52].

1.2.3 Immune system

During the foetal/neonatal period, the intestinal epithelial cells (IECs) play a key role in mucosal immunity by expressing various cytokines and cytokine receptors, such as tumor necrosis factor (TNF)-α, interleukin (IL) 1, IL-6, IL-8 and platelet activating factor [53]. IEC cells also express major histocompatibility complex (MHC) class II cell surface receptors, human leukocyte antigen I (HLA) and HLA-DR, by 18-22 weeks, and can serve, at least *in vitro*, as antigen presenting cells [54]. IEC HLA-DR expression may also be important in the maturation and selection of intra-epithelial lymphocyte clones [55]. Moreover, IECs can express a variety of non-classical MHC class I molecules, which may have a role in antigen presentation, as co-stimulatory molecules, or as intercellular adhesion molecules [56].

Foetal IECs are able to respond to lipopolysaccharide (LPS), through TLR4 and activation of the nuclear factor Kappa B (NF-κB), which may serve an important role in host defence (as this chemokine can recruit and activate both neutrophils as well as mononuclear phagocytes

[57]). The hyper-responsiveness of foetal enterocytes to LPS is due to lower levels of inhibitor of NF-κB [58]. NF-κB signals are down regulated after birth, perhaps as an adaptive mechanism to prevent inflammation from bacterial colonisation [59].

Enterocytes have an important role in immunoglobulin (Ig) transport. Enterocytes express Ig receptors and transporters which allows bidirectional Ig transportation, from the amniotic fluid (maternal IgG), and breast milk, across the epithelium and lumen during the foetal and neonatal period [57, 60, 61]. Other IECs also play an important role in host defence. Goblet cells start producing mucus by week 12 of gestation [33]. Paneth cells also appear at this time, and these cells produce antibacterial proteins such as lysozyme and α-defensins. The number of Paneth cells per crypt is developmentally regulated and increases with maturity until adulthood [62].

At birth, the major cellular components of the GIT mucosal immune system are present, but immature and inexperienced (Figure 1.1). The development of the protective functions of the GIT requires stimulation by the microbiota following colonisation with the maternal inoculum during passage through the birth canal [63, 64]. The immune system gathers experience as it is continuously faced with a myriad of antigens. The protective function of the GIT requires different factors to stimulate both innate and adaptive immune responses, in a complex and well regulated net of tolerance-inducing mechanisms residing in the gut-associated lymphoid tissue (GALT), the most extensive lymphoid system of the human body. GALT is formed by both inductive (Peyer's patches) and effector sites (lamina propria and sub-epithelial cells). During the first two weeks of postnatal life, however, when mature crypt-based Paneth cells are absent, neonatal mice IECs were shown to express a number of antimicrobial peptides, such as cathelicidin-related antimicrobial peptide. Cathelicidin expression decreases with weaning, at the same time that Paneth cells start to produce defensins [65]. Defensins work as a containment system that prevents the transit of external and unprocessed, potentially

harmful, antigens across the GIT barrier, but they are also constantly in contact with the microbiota and with food antigens.

Germ free (GF) rodent studies have revealed that the microbiota is one of the most important factors for the development of the GALT [66]. One of the key functions of GALT, known as oral tolerance, is to distinguish innocuous antigens from pathogenic micro-organisms and elicit an appropriate response. Food molecules and GIT microbiota are constantly sampled by the immune system through M cells, or directly absorbed through enterocytes or lamina propria dendritic cells (DCs) [67]. Lamina propria DCs, for instance, have long dendrites that can cross between enterocytes, directly sample antigens from the intestinal lumen and present them to naive T cells. Naive T cell responses to either food or bacterial signals are generally described in terms of two classes of mature T helper cells (Th) expressing the surface protein CD4 (CD4+ T cells), defined by their cytokine production: Th type 1 that modulate cell-mediated immunity by secreting INFγ and TNFα; and Th type 2 that modulate humoral immunity by secreting IL-4 and IL-6 [68]. The establishment of the tolerogenic mechanisms occurs through CD4+ T cells that suppress the expression of T effector cells (Th1 and Th2) and stimulate the expansion of lymphocyte regulatory T cells (TReg) by secreting cytokines such as IL-10 and transforming growth factor beta (TGF-β).

The increased diversity of the microbial community around weaning also coincides with dramatic changes in the morphology and function of the GIT. Changes in the absorptive capability of the SI enables the gradual transition from a high lactose milk diet as the primary source of carbohydrate to solid diets containing plant based carbohydrates [69, 70]. During the weaning period, withdrawal of maternal Ig present in the milk accompanys important changes in the mucosal immune system, such as an increase in IELs and lamina propria lymphocytes (LPLs) in the villi of the SI [71]. At weaning, the type of dietary carbohydrates that pass undigested into the large intestine changes; carbohydrates (oligosaccharides)

found in breast milk [72] are replaced by a mixture of complex polysaccharides of plant origin as a major source of carbon and nitrogen for bacterial fermentation [73]. This results in expansion of non-pathogenic commensal bacterial populations in the large intestine [74].

Products of bacterial fermentation, such as short chain fatty acids (SCFA) in the large intestine are also known to affect host metabolism. For example, butyrate has been shown to stimulate mucosal cell proliferation [68, 75] and has been shown to be a major energy substrate of the mucosa [68, 76]. Furthermore, SCFA, in particular butyrate, have been shown to induce beneficial immuno-modulatory effects, such as reducing *in vitro* pro-inflammatory cytokine production [77, 78], inhibition of intracellular signalling mechanisms following pro-inflammatory cytokine stimulation [79, 80], and stimulation of mucosal repair [81].

In addition to changes induced by SCFA production, increased direct bacterial contact with the mucosa due to proliferation of *Lactobacillus* and *Bifidobacterium* species can also affect GIT immune function. The interaction of cellular pattern recognition receptors to extracellular and intracellular bacterial structures, such as cell wall peptidoglycans, can trigger various signalling cascades that alter the profile of cytokine secretion in monocytes and IECs [82, 83]. Expression of the major pattern recognition receptors TLR and nucleotide-binding oligomerisation domain protein receptor (NOD) occurs primarily in IECs and antigen presenting cells such as macrophages and DCs [84]. IECs are, in most cases, the first line of contact between the commensal bacterial population in the GIT and the host defence system. Not only do they provide an essential barrier [85], but they are also thought to influence immune functions by directly or indirectly modifying DC, IEL and LPL activity [86-88]. *Lactobacillus* and *Bifidobacterium* species were also shown to promote T helper cell response, inhibiting a T cell imbalance (Th2 > Th1 or Th1 > Th2) that may lead to disease [89]. These findings suggest that GIT microbial composition might affect immune homeostasis and the balance between microbiota, immune response and tolerance

mechanisms essential for a healthy GIT. Biased colonisation may result in diet intolerance in early postnatal life and in GIT disease in childhood [89].

Manipulation of the GIT microbiota in early life may provide a window of opportunity to have long lasting effects on the microbial community composition and activity, and consequently host health. Indeed, this concept is supported by differences in faecal microbiota composition and reduced microbiota diversity of infants associated with increased incidence of atopic eczema and obesity in later life [90-92].

1.3 Microbiota

The GIT microbiota in humans is highly complex and comprises many hundreds of bacterial species (or phylotypes) [93]. Bacterial density is different in each part of the GIT. The stomach and proximal SI contain relatively low numbers of microbes (10^3–10^5 bacteria/g or mL content) because of low pH and rapid flow in this region. Acid-tolerant lactobacilli and streptococci predominate in the upper SI. The distal SI (ileum) maintains a more diverse microbiota and higher bacterial numbers (10^8/g or mL content) than the upper SI and is considered a transition zone preceding the large intestine [94, 95]. The large intestine (colon) is the primary site of microbial colonisation because of slow substract and microbe turnover and is characterised by large numbers of bacteria (10^{10}–10^{11}/g or mL content), low redox potential, and relatively high SCFA concentrations [93, 96].

The pattern of colonisation of the large intestine and the other parts of the GIT can have both short term and long term health effects [91, 97-100]. Bacterial colonisation of the GIT is important for several reasons: bacteria or bacterial products provide essential nutrients for the infant (such as vitamins and SCFA), they stimulate the development of the immune system (especially adaptive responses), and they provide general protection against pathogen colonisation, as well as other functions [101, 102]. The GIT microbiota have a role in the onset of over 25 diseases or syndromes [103], such as obesity and type 2 diabetes

[104]. The first colonisers of the GIT are facultative anaerobic bacteria, such as *Escherichia coli*, enterococci and streptococci, which predominate in the first days of life [105]. These bacteria consume oxygen in the GIT lumen, creating an anaerobic environment more favourable for the proliferation of strict anaerobes, such as *Bacteroides*, *Clostridium* and *Bifidobacterium* species [106]. The presence of the first colonisers orchestrate the succession of bacteria [107], where the composition depends on a diverse number of factors such as mode of delivery, type of feeding, and genetic, cultural and geographical determinants [108]. Given the potential impact the GIT colonisation has on subsequent health and disease [103], there has been much interest in studying the factors that determine the establishment of the first GIT microbiota.

The three main stages of the initial GIT colonisation are: prepartum, partum and postpartum.

1.3.1 Prepartum

Historically, the human foetus has been considered microbiologically sterile, with the first microbial exposure taking place at vaginal birth through contact of the newborn with the maternal vaginal and GIT microbiota, and the surrounding environment [93]. However, in the last decade, discoveries point to pregnancy as the beginning of bacterial exposure for the developing foetus [109-111]. Bacteria from the maternal GIT and/or urogenital region, such as *E. coli*, *Staphylococcus* spp, *Enterococcus* spp. and *Lactobacillus* spp., have been isolated and/or detected in umbilical cord blood [111], amniotic fluid [112], meconium [110], placental [109] and foetal membranes [109, 113, 114] without any clinical evidence of infection or inflammation in the mother-infant pair (Figure 1.2). However, there is a delay before the meconium can be harvested (up to several days after birth), and this is more than enough time for the mother's commensal microbiota (from the vagina or the skin) to reach the infant GIT, suggesting a very early rather than prenatal colonisation. Moreover, the presence of bacteria in the amniotic fluid could be an indication of an undetected infection

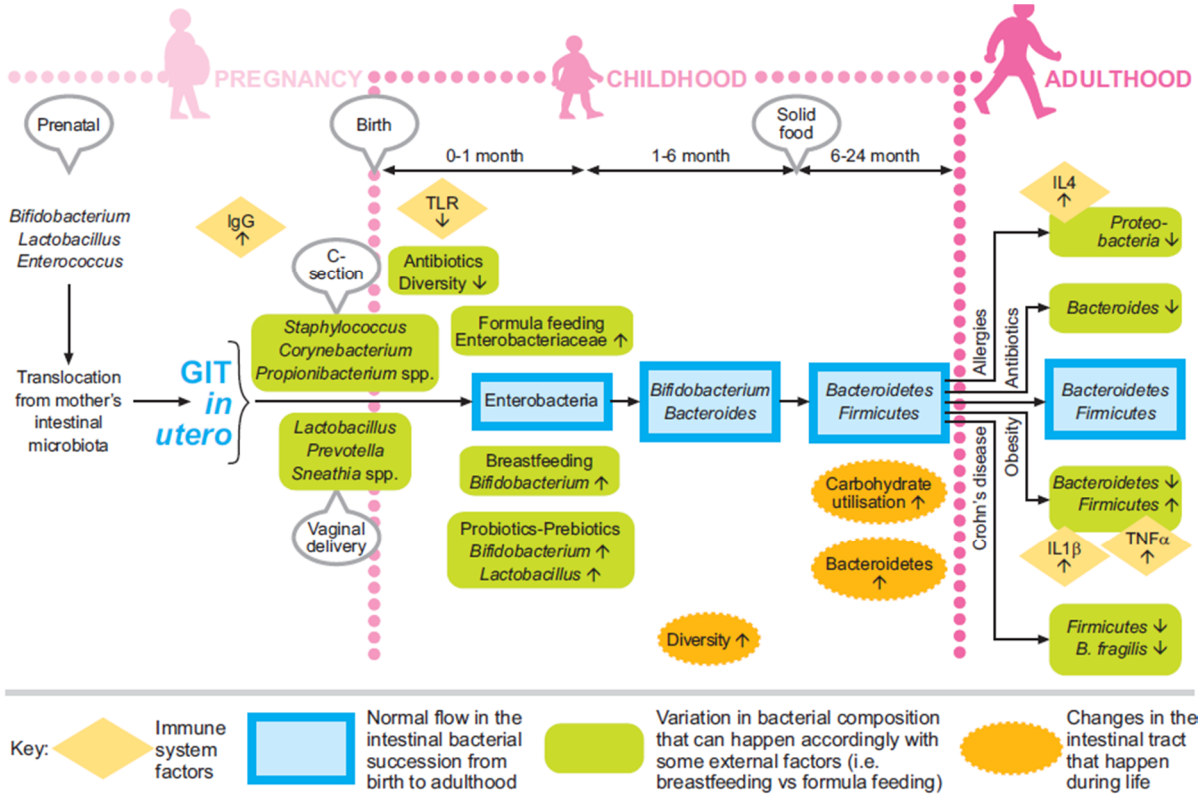


Figure 1.2 Development of the gastrointestinal tract microbiota in infants and changes associated with health and diet, with rectangles indicating predominant bacterial groups at a particular stage in life. Initial community structure is influenced by maternal gastrointestinal tract microbiota, delivery mode and type of feeding. Diversity increases as the infant grows and begins eating solid food. Different disease states, diets and environmental conditions all play a role in shaping the microbiota throughout life. Figure adapted from Clemente *et al*, 2012, [115].

and increases the risk of preterm labour [116]. These results raise more questions than they answer.

It is still unknown if the presence of bacteria in the intrauterine environment is systematic or exceptional, whether these bacteria are viable and capable of colonising the infant GIT, and what influence they can have on later stages of the development of the infant GIT microbiota. Rather than an inoculum for GIT colonisation, the low levels of bacteria detected in umbilical cord blood, amniotic fluid, placenta and foetal membranes are likely to stimulate the development of the foetal GIT mucosal immune system [117], preparing it for life outside the uterus. Supporting this, microbial RNA and unmethylated DNA cytosine phosphate guanine (CpG) islands were shown to exert immune regulatory effects in adult GF mice, acting through TLR 9 [118-120]. The absence of adverse neonatal immune responses after microbial exposure in the birth canal also supports the existence of complex regulatory mechanisms that are likely to commence during foetal development [121].

In summary, it is still unclear whether colonisation of the infant GIT starts *in utero*, however there is some indication that the bacteria or their components, present in the foetal membranes during pregnancy, may contribute to immunological stimulatory and protective or depletory effects for the neonate, and may be closely associated with maternal GIT and/or urogenital microbiota [122].

1.3.2 Partum

Mode of delivery (vaginally or by caesarean section) has been demonstrated to have a strong influence on early GIT colonisation, particularly on the number of *Bifidobacterium* sp. [84, 123]. Analysis of the meconium of newborn infants by pyrosequencing revealed a strong correlation between the first microbial communities of the GIT and the microbial communities of either the mother's vagina (*Lactobacillus*, *Prevotella*, or *Sneathia*) for vaginal delivery, or the mother's skin (*Staphylococcus*, *Corynebacterium*, and *Propionibacterium*) for caesarean

section [124]. Temporal temperature gradient gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE) analyses of faecal samples 3 days after birth also revealed differences between caesarean and vaginally delivered infants. *Bifidobacterium* numbers and overall microbial diversity was lower in caesarean than in vaginally delivered infants [123] This demonstrates that the GIT environment becomes populated by the first abundant microbial communities it encounters, either the skin or the vagina. The causal relationship between caesarean delivery, the shift in microbiota and the link to many childhood diseases has been recently reviewed by Neu *et al.* [125]. The authors suggested that, to understand the effects of caesarean delivery on microbial colonisation and childhood diseases, a better method to categorise the indication of caesarean is needed. A foetus delivered by caesarean after being exposed to 8 centimetres dilation during a long labour, for example, would be susceptible to a different microbial environment than a foetus delivered by caesarean because of maternal request (i.e. before rupture of membranes).

Gestation time is also a factor that strongly influences the establishment of the infant GIT microbiota after delivery. Enterobacteriaceae and other potentially pathogenic bacteria such as *Clostridium difficile* or *Klebsiella pneumoniae* were found in greater numbers in preterm infants when compared to full term infants. In full term infants, the diversity of the faecal microbiota was higher, and more common genera such as *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* were present [126].

1.3.3 Postpartum

The succession of bacterial species present after delivery is complex, and presumably influenced by the mode of feeding (breast milk or formula). Human milk is associated with long term health benefits. The World Health Organisation recommends exclusive breastfeeding for six months, and supplemental breastfeeding for up to two years or longer. Although the worldwide prevalence of exclusive breastfeeding for the first four to six months of life has increased (41% at 4 months and 25% at 6 months), rates are lower for many

developed countries [127], and as such, many infants are formula fed for most of their first months of life. Although attempts have been made to improve formula quality, differences in GIT microbial colonisation and susceptibility to GIT disease or sepsis are still observed between breast fed and formula fed infants [128, 129]. By using high throughput sequencing and biochemical analyses, a recent study showed a significant enrichment of Actinobacteria and Firmicutes, and depletion of Proteobacteria in breast fed compared to formula fed infant faeces. Enterobacteriaceae (Proteobacteria) were the dominant bacteria in formula fed infant faecal microbiota, and Veillonellaceae (Firmicutes) and Enterobacteriaceae (Proteobacteria) were dominant in the breast fed infant faecal microbiota [128]. Breastfeeding is also associated with a lower risk of being overweight and obese (at least in childhood), lower levels of arterial blood pressure, lower total- and low-density lipoproteins and cholesterol levels in adulthood, and lower risk of developing type 2 diabetes [130, 131].

1.4 Breast milk

After birth, the profound and intimate connection between a mother and her new born continues through breastfeeding. Breast milk is the physical representation of this relationship: synthesised at the mother's expense, and shaped throughout evolution to nourish the infant and to maximise its rate of survival. Human milk is perhaps the most personalised food, both in its nutritional and in the non-nutritive composition, where the molecular make up varies from mother to mother and across lactation stage, providing the infant all the nutrients needed in a concentrated form [132].

1.4.1 Nutritive components

Human milk is a complex food, and its composition reflects all the nutritional and physiological demands of the newborn. The macronutrients in human milk, such as lactose, fat, and proteins, are absorbed by the SI at a rate that is limited by the development and maturation of the GIT [32]. The macronutrient composition of human milk varies within

mothers and across lactation, but is remarkably conserved across populations despite variations in maternal nutritional status (Table 1.1) [133]. Other micronutrients present, such as nucleotides, vitamins and minerals, are also highly available for the infant.

1.4.2 Macronutrients and micronutrients

Human milk proteins can be divided into the whey and casein fractions or complexes, each comprising a remarkable array of specific proteins and peptides. The most abundant proteins are caseins, α-lactalbumin, lactoferrin, sIgA, lysozyme, and serum albumin [134]. Non-protein nitrogen containing compounds, including urea, uric acid, creatine, creatinine, amino acids, and nucleotides, comprise approximately 25% of human milk nitrogen [135]. Human milk fat is characterised by high ratios of palmitic and oleic acids, the former heavily concentrated in the 2-position and the latter in the 1- and 3-positions of the triglycerides. Fat is the most variable macronutrient of milk. Hind milk, defined as the last milk of a feed, may contain 2 to 3 times the concentration of milk fat found in fore milk, defined as the initial milk of a feed [136]. A study of milk from 71 mothers over a 24 hour period found that the milk fat content was lower in night and morning feedings than in afternoon and evening feedings [137].

The fatty acid profile of human milk varies with maternal diet, particularly the long chain polyunsaturated fatty acids (PUFA). Intake of PUFA in the Western world is skewed toward the n-6 PUFA, with suboptimal intake of n-3 PUFA [138]. The docosahexaenoic acid (n-3 PUFA) composition of human milk is particularly low in North American populations, where supplementation should be considered for breastfeeding North American women on docosahexaenoic acid limited diets[139].

The principal carbohydrate in human milk is the disaccharide lactose. The concentration of lactose in human milk is the least variable of the macronutrients, but higher concentrations of

Table 1.1 Nutritional composition of human milk in early lactation [140, 141].

Energy content (kcal/dL)		63.3
Macronutrients (g/dL)		
Protein		1.9
Lipid		2.9
Carbohydrates	Lactose	7.3
	Oligosaccharides	0.5-1.5
Micronutrients (mg/dL)		
Calcium		30
Chlorine		69
Copper		48
Iron		69
Manganese		1.9
Phosphorous		17
Potassium		62
Sodium		30
Zinc		0.4

lactose are found in the milk of mothers producing higher quantities of milk [142]. Other significant carbohydrates in human milk are the oligosaccharides, which comprise approximately 5-23 g/L in human milk, depending on the stage of lactation and maternal genetic factors [143]. Oligosaccharides are among the non-nutritive bioactive factors discussed in section 1.3.3.1.

1.4.3 Non-nutritive components

Human milk also provides non-nutritive components (e.g. Ig, lactoferrin, lysozyme and oligosaccharides) to the offspring that facilitate the adaptive, functional changes required for optimal transition from intrauterine to extrauterine life [144-146]. A common characteristic of these components is that they are glycosylated molecules [147] as glycosylation confers protection against GIT digestion. Glycans in milk can be found as free human milk oligosaccharides (HMO), or conjugated via glycosidic bonds to proteins or lipids.

1.4.3.1 Oligosaccharides

Human milk is a rich and natural source of carbohydrate oligosaccharides (5 to 23 g/L), being the third most abundant constituent in terms of concentration after lactose and lipids [148]. HMO cannot be digested by the human GIT, and remain intact until they reach the large intestine, while having no apparent direct nutritional role, HMO are involved in promoting the GIT colonisation, bacterial metabolism [149] and subsequent signalling between commensal bacteria and their host. These interactions play pivotal roles in the development of the mucosal immune system and prevention of disease [150]. Lactation could be considered the second step of “immunological education”.

Small amounts of HMO, lacto-N-tetraose and its monofucosylated derivative lacto-N-fucopentaose II, can be found in the infant urine [151], suggesting that these molecules can exert physiological effects, not only in the GIT, but also systemically. The myriad of functions attributed to HMO are probably due to the variety of their functional structures. HMO consists

of a pool of soluble carbohydrates with a degree of polymerisation (DP) of 3 to 15, linked through a variety of glycosidic bonds [152, 153] (Figure 1.3). HMO are composed by combinations of five monosaccharides: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and N-acetylneuraminic acid (NeuAc; sialic acid). All HMO are characterised by a terminal lactose molecule, modified by fucose or sialic acid in the case of the shorter HMO such as 2'-fucosyllactose (FL), 3FL and sialyllactose (SL), or by repeats of building blocks of lacto-N-biose type 1 (LNB; Galb1-3GlcNAc) or N-acetyllactosamine (LacNAc; Galb1-4GlcNAc). These repeats can be further decorated by fucose or sialic acid in α-linkages, adding more complexity and diversity to these molecules [154]. HMO can be classified as acidic or neutral depending on the presence of the negatively charged sialic acid. Neutral HMO can be further categorised by the presence of fucose on their structures. More than 200 molecular structures differing in size, charge, and sequence have been identified in human milk samples [152, 153].

HMO vary qualitatively and quantitatively with the maternal Lewis blood group and secretor status [155], which generates four different groups of milks (Figure 1.4) [143]. Additionally, lactation stage, diet, lifestyle, ethnicity and other factors also contribute to structural variations of HMO [155]. The complexity of the carbohydrates in breast milk may influence the level of protection transferred to the offspring. It is likely that infants raised in particular environments, or during specific growth stages or physiological states, take advantage of selective variations in the amounts and structural differences of HMO. For example, it has been shown that HMO from individuals with Lewis blood group B exhibit preferential binding to pathogens, especially *Helicobacter* pylori [142], and children with Lewis blood group A have increased susceptibility to enterotoxigenic *E. coli* diarrhoea [143].

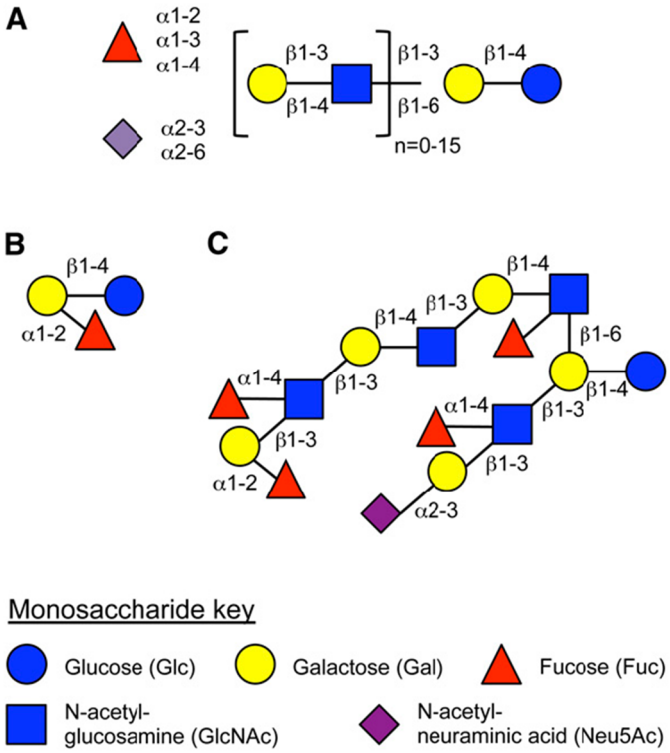


Figure 1.3 Human milk oligosaccharide structures. (A) Structural composition of HMO. If n = 0, the lactose backbone is either sialylated or fucosylated to form human milk trisaccharides such as 2'-fucosyllactose (B). If n > 0, complex HMO are formed that can be branched and also modified like the sialylated and fucosylated iso-lacto-N-decaose (C). The monosaccharide key is shown on the bottom of this figure. Figure extracted from Bode, *et al.*, 2012, [154]. HMO, human milk oligosaccharides.

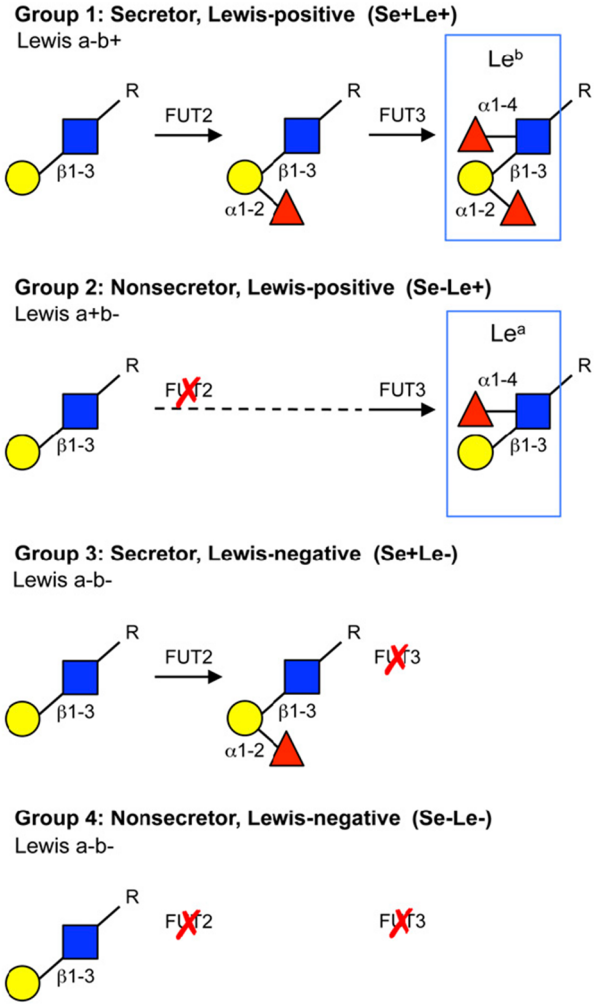


Figure 1.4 Genetically determined variations in HMO. Composition of HMO fucosylation highly depends on the expression of the secretor gene that encodes the α1–2-fucosyltransferase FUT2 and the Lewis gene that encodes for the α1–3/4-fucosyltransferase FUT3. Based on the expression of these fucosyltransferases, four different HMO groups can be distinguished. If both fucosyltransferases are expressed, the milk contains oligosaccharides with the Lewis group B (Le^b). If FUT3, but not FUT2, is expressed, the milk contains oligosaccharides with the Lewis group A (Le^a). Neither Le^a nor Le^b epitopes are present on HMO of women that do not express FUT3, but the HMO profile can be different depending on whether or not FUT2 is expressed. HMO, human milk oligosaccharides; Le^a, Lewis a blood group antigen; Le^b, Lewis b blood group antigen. Figure extracted from Bode, *et al.*, 2012, [154].

1.3.3.2 Microbiota

A minor component of breast milk is bacteria. Culture dependent methods have long confirmed the presence of bacteria, including *Staphylococcus*, *Streptococcus* and *Bifidobacterium* species, in aseptically collected human milk [156, 157]. Culture independent studies using characterisation techniques based on the amplification of bacterial 16S rRNA have shown that human milk contains several additional bacterial genera, including *Lactobacillus* and *Enterococcus* species [157-159]. Recently, pyrosequencing of 16S rRNA genes in human milk has suggested a core microbiome consisting of 9 bacterial genera - *Staphylococcus, Streptococcus, Serratia, Pseudomonas, Corynebacterium, Ralstonia, Propionibacterium, Sphingomonas and Bradyrhizobiaceae* [160]. Although this technique is widely accepted to determine microbial diversity, it does present limitations, such as a lack of information on the functional capacity of the microbes within the milk matrix, and it doesn't assess the types of DNA motifs to which an infant is exposed. Other studies using cultivation and quantitative real time PCR have shown greater levels of complexity and individuality in the milk microbiota when compared to the proposed core microbiome [157-159, 161]. The human milk microbiome was also shown to change over time, and may be dependent on the mother's weight and how the infant was delivered [162].

Ingestion of viable bacteria in human milk may lead to effective colonisation and development of the infant GIT, as shown by studies where microbial composition of breast milk was similar to that in infant faeces [163, 164]. The presence of bacterial DNA alone may also stimulate the infant immune development. For example, CpG dinucleotides within bacterial DNA are known as potent immune stimulators, acting through toll-like receptor 9 [120]. Conversely, immune suppressive motifs including poly-guanosine or guanosine cytosine-rich sequences, such as those on the telomere region of mammalian DNA, that can block immune activation induced by CpGs [165]. Recently, immune suppressive motifs (TTAGGG and TCAAGCTTGA) that are able to counter the effects of CpGs have been

discovered in *Lactobacillus* [166]. If immune modulatory motifs occur in human milk-derived DNA, they could contribute to the immune development by decreasing exaggerated inflammatory responses to colonising bacteria, which are seen in infants with necrotising enterocolitis [167]. The exposure to the bacteria in milk may educate the infant immune system through PRR CD14 and TLR [168] that recognise bacterial cell wall components. These receptors may induce analogous responses to maternal antigens and protect the neonate against pathogens [169, 170]. The maternal GIT may be the origin of bacteria found in umbilical cord blood and amniotic fluid [111, 171, 172] as well as in human milk [173] (Figure 1.5). Experiments with pregnant mice orally administered a labelled *Enterococcus faecium* strain showed that the label could be detected in umbilical cord blood [111] and meconium [110] of the foetus obtained by caesarean section.

The mechanisms of maternal bacterial transfer to the foetus and milk are unclear. It has been proposed that maternal DC and leucocytes play an important role in the bacterial uptake into placenta and milk (Figure 1.5) [169]. $CD103^+$ DC in the Peyer's patch can cross the paracellular space of the GIT epithelium to take up bacteria directly from the lumen [174]. Once internalised by DC and/or macrophages, bacteria can spread to other locations, such as the respiratory and genitourinary tract, salivary and lachrymal glands, and most significantly, the lactating mammary gland, via lymphatic and blood circulation [173, 175]. DC are relatively ineffective at killing internalised organisms [173, 175] and may be responsible for viable bacteria reaching the mammary glands and placenta [169, 173, 176, 177]. Once in the blood circulation, maternal bacteria may be transferred to the foetus via the paracellular pathway of the placental barrier [178]. This is analogous to the transfer of extracellular pathogens shown to occur across the blood-brain barrier [179] including *Streptococcus pneumoniae* and group B streptococci; bacteria closely related to the predominant species found in human milk. Beginning prepartum and persisting throughout

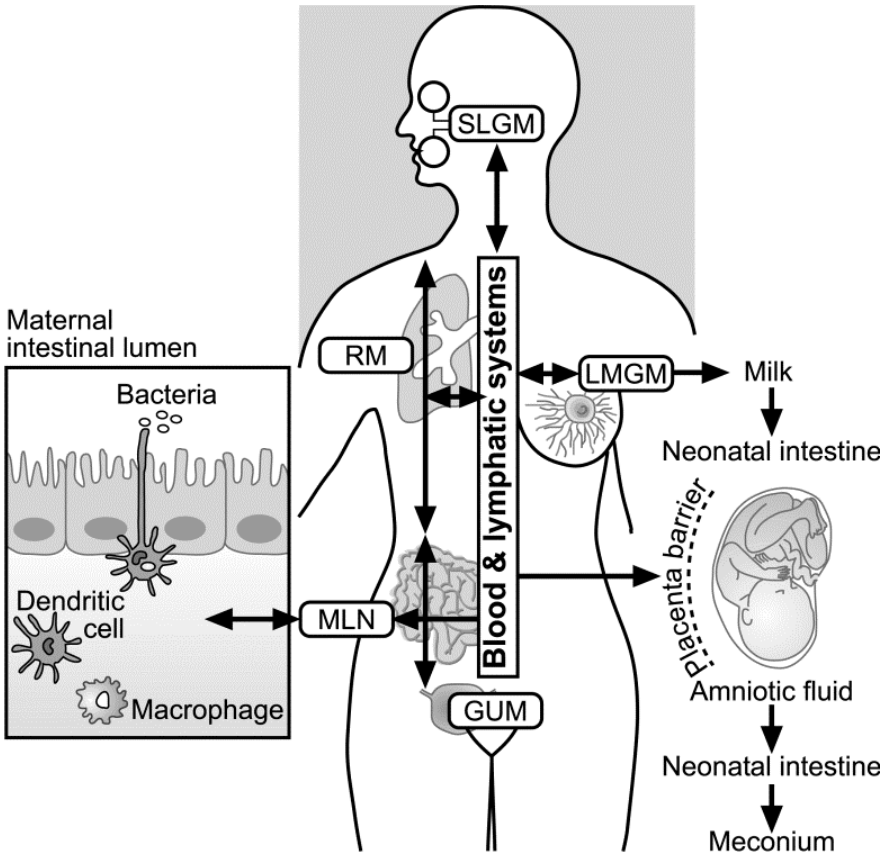


Figure 1.5 A hypothetical model to explain how maternal microbiota and microbial products could be transferred from mother to the foetal and neonatal gastrointestinal tract. Dendritic cells can cross the paracellular space of the gastrointestinal tract epithelium to take up bacteria directly from the lumen. Circulation of lymphocytes within the mucosal associated lymphoid tissue allows the maternal gastrointestinal tract microbiota to reach distant mucosal surfaces, including those found in the respiratory and genitourinary tracts, salivary and lachrymal glands and lactating mammary gland. Genitourinary tract mucosa, GUM; lactating mammary gland mucosa, LMGM; respiratory tract mucosa, RM; salivary and lacrimal glands mucosa, SLGM; mesenteric lymph node MLN. Figure adapted from Martin *et al*., 2004, [161].

the postpartum period, the maternal immune system shifts from cell-mediated immunity [180] towards one characterised by humoral immunity [181]. This altered maternal immunological state may increase the translocation of bacteria and their components from the maternal GIT into the blood and lymphatic circulation [173]. Near term, thinning of the placental barrier increases nutrient, waste product and gas exchange efficiency between mother and foetus [182] and possibly contributes to the influx of commensal bacteria present in the blood circulation. This influx may initiate the first adaptation of the foetal GIT for life outside the mother.

1.5 Maternal nutritional supplementation

Pregnancy and lactation is a period characterised by maternal physiological changes and intense foetal and neonate growth and development. Consequently, consumption of foods that supply adequate amounts of nutrients to meet maternal and foetal requirements are generally greater than during other life stages [183]. The recommended dietary intakes of 14 out of 21 essential micronutrients (7 vitamins, 5 minerals, and choline), for example, increase during pregnancy [184]. Although meeting these increased nutrient requirements can and probably should be achieved by the consumption of appropriate amounts of foods in a balanced and varied diet, it is not always possible, and the use of dietary supplements may be recommended. A recent systematic review [185], on the effect of supplementing different types and combinations of micronutrients to pregnant women evaluated 17 articles in the field. Calcium supplementation, for example, was shown to protect against pre-eclampsia [186-188]. Multiple micronutrients supplementation during pregnancy was shown to reduce low birth weight more compared with the usual iron-folate supplements. The authors concluded that supplementation of micronutrients (mainly multiple micronutrients), has beneficial effects in reducing the risk of low birth weight and other complications, though further studies were also recommended.

The maternal GIT tract is a potential target for prenatal supplementation. Changes in maternal GIT microbiota composition, for example, have been associated with alterations to biochemical parameters in the maternal blood (e.g. increased folic acid and ferritin levels, and reduced transferrin and cholesterol levels), with possible consequences for pregnancy progression, foetal programming, and newborn health [189, 190]. The presence of *Actinomyces naeslundii* or gram-negative anaerobes in the maternal oral cavity has been associated with earlier delivery and lower birth weight, while the presence of lactobacilli has been linked to term delivery and heavier birth weight [171, 191, 192]. Fak *et al*. [193] evaluated the effects of maternal GIT microbiota changes on offspring health by orally treating pregnant rats with live non-pathogenic *E. coli* (strain CCUG 29300T) isolated from human urine (cystitis) before delivery, or with broad spectrum antibiotics before delivery and during lactation [193]. Compared with the offspring from untreated mothers, offspring from dams of both treatments had higher concentrations of *Enterobacteriaceae*, which correlated with decreased stomach growth and function, lower total pancreatic protein levels, higher SI permeability, and increased plasma levels of the acute phase protein, haptoglobin [193]. These findings suggest that specific components of the maternal GIT microbiota, or maternal treatment with broad spectrum antibiotics, may influence the diversity of the offspring GIT microbiota and the development and maturation of the offspring mucosal immune system [194, 195].

Many factors affecting the initiation and course of auto-immune diseases appear to act within a narrow window of development (pregnancy and early life [141, 196]). Maternal immune status and GIT composition seem to affect infant GIT microbiota, as well as the incidence of allergic diseases. Allergic mothers (with history of atopic eczema, allergic rhinitis, asthma), for example, had lower numbers of bifidobacteria in their breast milk and faeces, and there were decreased counts and reduced diversity of bifidobacteria in the offspring faeces [197], compared with non-allergic mothers. The establishment of specific microbiota in infants,

such as bifidobacteria [198], has been shown to alter the signalling which determine T-cell differentiation and/or the induction of tolerance [199, 200].

Maternal exposure to environmental stimuli, particularly bacteria and antigens prenatally, appears to play an important role in postnatal immune responsiveness and the subsequent development of auto-immune disorders [141, 196, 201]. For example, cord blood from neonates born to farming families was shown to contain higher levels of interferon gamma (IFNγ) and TNF-α compared with cord blood from infants born to non-farming families [202]. Decreased levels of IFNγ in cord blood at birth was associated with late onset of allergies in adult life, which might indicate that this cytokine plays an important role in regulating the effects of maternal environmental stimuli to the developing foetal immune system [203]. Maturation of the adaptive immune system and development of functionally active T-cells has been shown to start *in utero*, influenced by environmental factors such as bacterial exposure, and is a critical phase in foetal programming of the offspring [204].

Overall, the maternal GIT microbiota appears to influence pregnancy progression and contributes to microbial colonisation of the new born GIT [205]. A maternal GIT microbiota rich with bifidobacteria and with fewer *E. coli* may contribute to improving foetal and/or neonatal development and maturation, as well as decreasing the incidence of immune disorders in infants. Dietary manipulation of maternal GIT microbiota during the peri- and postnatal period may be an important method for improving offspring health and later life outcomes.

1.5.1 Probiotics

It is well known that administering probiotic bacteria to adults alters the GIT diversity [206] and provides health benefits [207, 208]. Specific lactobacilli and bifidobacteria species have traditionally been recognised as potential health promoting microbes in the human GIT and are, broadly used as probiotic supplements in foods. The beneficial effects of lactobacilli and

bifidobacteria consumption are likely to involve inhibition of pathogen adherence to the mucosa, improvement of barrier function of the GIT epithelium, production of bacteriocins, increased mucosal IgA production and reduced mucosal proinflammatory cytokine secretion [209]. Probiotic dairy products containing *Lactobacillus* and bifidobacteria have been shown to reduce the risk of spontaneous preterm delivery [210] and preeclampsia [211], as well as increasing serum levels of erythrocyte glutathione reductase [212] (an enzyme involved in cellular antioxidant activity), when consumed by mothers during pregnancy. Probiotics also enhance disease resistance in infants [213, 214]. However, to be effective, probiotics must be continually taken because they do not persist in infants [215, 216] or adults [217] after administration is discontinued.

Evidence that maternally derived probiotic bacteria colonise the GIT of vaginally delivered and breast fed infants, and persist for 1-2 years, comes from studies analysing the effects of perinatal *Lactobacillus rhamnosus* GG supplementation on the development of allergic disorder in the offspring [205, 218-220]. Blumer *et al.* [221], for example, reported that pregnant mice supplemented with *L. rhamnosus* GG had an altered placental pro-inflammatory cytokine expression, with lower IL-4 and increased TNF-α gene expression levels. This was associated with reduced allergic airway inflammation in the offspring [221]. Similarly, in a human intervention study, maternal *L. rhamnosus GG* supplementation increased cord blood and breast milk levels of anti-inflammatory cytokines, IFNγ and TGF-β1 in the first week, when compared with the placebo group [222]. A Finnish study also showed that maternal pre- (1 month) and postnatal (6 months) supplementation with *L. rhamnosus GG* reduced the frequency of eczema in the offspring at 1, 2, 4, and 7 years of age, but had no effect on atopic sensitisation [213, 223-226]. Administration of *L. rhamnosus GG* to mothers during pregnancy was able to decrease plasma levels of immunoglobulin E (IgE) antibodies to a mixture of food allergens in infants up to 2 years of age [227]. The same treatment increased colonisation by particular *Bifidobacterium* species [218, 228], but failed

to modulate the microbial diversity of 1 week old infants GIT [229]. In a human-like atopic dermatitis model using NC/NgaTnd mice, perinatal administration of *L. rhamnosus GG* also decreased clinical symptoms of dermatitis, scratching frequency and plasma IgE levels, and increased levels of IFNγ in skin biopsies [208].

Combined probiotic intervention with *L. rhamnosus GG* and *Bifidobacterium lactis* Bb12, together with dietary counselling, has been shown to ameliorate glucose homeostasis in healthy young females during and after pregnancy [230]. Probiotic intervention also reduced the frequency of gestational diabetes mellitus (13% incidence for diet/probiotics v. 36% diet/placebo and 34% control groups) [231]. The same maternal treatment and dietary counselling regime reduced the proportion of infants with a high 32-33 split proinsulin (a well characterised metabolic marker of insulin resistance) when compared with control/placebo groups [232]. Kaplas *et al*. [233] showed that a combination of a balanced diet and probiotic therapy with *L. rhamnosus GG* and *Bifidobacterium lactis* Bb12 was able to increase placental concentration of phospholipid fatty acids, which are known to exert immunomodulatory effects on the foetus later in pregnancy [234, 235]. Increased concentrations of fatty acids available to the foetus reduced food allergy risks and IgE associated eczema in infants during the first year of life [236], and a lowered the rate of allergic asthma in 16 year old children [237]. These findings suggest that the beneficial effects of these probiotic strains may, at least in part, be mediated via the placenta through induction of immunoinflammatory signals and may promote neurological development at an earlier age [238], as well as reduce the risk of a range of immunoinflammatory disorders [233].

There is, however, insufficient scientific evidence to support many of the health claims attributed to probiotics. As only a limited number of strains, such as bifidobacteria and lactobacillus, have been extensively studied for possible health benefits, other species (such as *Streptococcus thermophilus* [239]), already sold as probiotics, should be investigated.

Concerns exist about the overall safety of administering probiotics to high risk patient groups, including pregnant women, preterm neonates and infants [240]. Invasive infections caused by probiotics translocated into the blood stream, have been reported in patients with immune dysfunction/suppression or with an abnormal GIT epithelial barrier [241, 242]. There is also a risk of transferring antibiotic resistant plasmids from some probiotic organisms [243].

1.5.2 Prebiotics

The addition of oligosaccharides resistant to digestion in the SI (prebiotics) to the diet of pregnant mothers to influence the maternal GIT microbiota population for the benefit of the neonate may be a safer alternative to probiotic supplementation [244]. Oligosaccharides affect GIT function by selectively stimulating the growth and/or activity of beneficial bacteria such as bifidobacteria and lactobacilli, thus improving host health [245]. Fujiwara *et al*. [27, 246] demonstrated that maternal dietary supplementation with fructo-oligosaccharide (FOS) changed the GIT microbiota of the offspring, and diminished the severity of atopic dermatitis. In contrast, the combined dietary supplementation of galacto-oligosaccharides (GOS) and FOS changed the maternal microbiota, but the effect was not transferred to the offspring [244]. Another study showed that a GOS and inulin enriched diet, fed throughout pregnancy and lactation, increased colon length and thigh muscle mass in the offspring [247].

Consumption of prebiotics during pregnancy may also increase the production of bacterially-derived metabolites, providing benefits for the woman and her offspring. Inclusion of inulin and GOS in the diet during pregnancy increased the number of bacteria synthesising folate, the levels of folate in the digesta of the large intestine and consequently, the levels of folate in the blood stream available for foetal development [248]. Suboptimal concentrations of folate during pregnancy have been associated with anaemia, neural tube defects, vascular disease, neuropsychiatric disorders, and cancers [249]. Another group of metabolites produced by microbial fermentation that may affect the foetus during pregnancy are SCFA.

Most SCFA produced by colonic bacteria (predominantly acetate, propionate and butyrate), are absorbed by colonic epithelial cells and metabolised, contributing to the mother's overall energy supply (and that of the developing foetus [250]). Butyrate is also a histone deacetylase inhibitor that is believed to activate genes by increasing histone acetylation and decreasing DNA methylation [251]. There is evidence that SCFA mediated histone deacetylase inhibition may play a part in gene regulation of foetal globin [252] and haemoglobin [253], and that this, in turn, may have a role in the normal regulation of human γ- to β-globin gene switching, important in the formation of haemoglobin.

1.5.3 Synbiotics

Recently, co-therapy of probiotics and prebiotics (synbiotics) was tested in pregnant women and their infants to assess possible effects in preventing allergic diseases [254, 255]. 1223 pregnant women carrying infants with a high risk of developing atopic dermatitis were randomly assigned to either a daily mixture of four probiotic strains with GOS, or a placebo for two to four weeks before delivery. After delivery, infants either received the same probiotic mixture with GOS or the same placebo as the mother for up to 6 months. Synbiotic treatment showed no effect on the incidence of respiratory allergic diseases by 2 years of age, but prevented atopic eczema, increased resistance to respiratory infections and reduced IgE-associated atopic dermatitis [254, 255]. In the same study, the synbiotic treatment improved the response to *Haemophilus influenzae* type b (Hib) immunisation, increasing Hib antibody concentrations compared with the placebo group without impairing antibody responses to Hib, diphtheria or tetanus.

The effects of synbiotic supplementation on the immunological composition [256] and total antioxidant capacity [257] of breast milk have recently been investigated. Maternal synbiotic supplementation was able to increase milk IgA and TGF-β2 levels. The incidence of diarrhoea in infants also decreased in the supplemented group, while no changes were observed in the placebo group after the experimental period. The authors suggested that

synbiotic supplementation may have positive effects on the immune composition of breast milk and the reduction of diarrhoea incidence in infants. The total antioxidant capacity and malondialdehyde levels (a biomarker for oxidative stress), of human breast milk were evaluated after daily synbiotic supplementation of lactating mothers for 30 days [257]. Antioxidant capacity of breast milk was shown to be higher in the supplemented group compared to the placebo group. Although the malondialdehyde levels decreased in the supplemented group, it increased in the placebo group after the experimental period. Synbiotic supplementation may have positive effects on the antioxidant capacity and malondialdehyde levels in breast milk, however, these findings require confirmation from future studies.

A mixture of pro- and prebiotics has a synergistic effect by stimulating the growth and/or metabolism of the delivered probiotic bacteria, inducing, for example, the production of SCFA that have direct anti-pathogenic and immune modulating effects [258]. It is difficult to define the specific mechanism underlying the functional effects of pro- and prebiotics, due to the complexity of the GIT microbiota and its interaction with the host's mucosa and immune system. However, the reported relationship between allergic disease and the composition of the GIT microbiota early in life points out the prenatal and perinatal periods as an opportunity to improve offspring health through maternal supplementation.

1.5.4 Alternative sources of milk oligosaccharides for the mother/infant

The prebiotics GOS and FOS have been used to improve human health, and have been the only oligosaccharides tested that target infant health through maternal supplementation. Although the supplementation of maternal diet and infant formula with GOS and FOS has shown promising results in terms of enriching bifidobacteria, several questions remain. HMO, for example, selects restricted bifidobacteria (primarily *B. infantis* and *B. bifidum* species), that possess the requisite genetic capacity (in particular fucosidase and sialidase functions), to deconstruct the HMO polymer. In contrast, FOS and GOS are more broadly

used across the genus, leading to non-specific microbial enrichment of the GIT. HMO also possess many functions (related to their different structures), that benefit the infant's GIT development and maturation [259]. It is unlikely that the simple structures found in FOS and GOS possess similar developmental, immunological, or antiadherence functions to the infant.

Human milk is obviously not amenable to large scale production, but there is an urgent demand for alternative, functionally comparable, oligosaccharide sources to supplement infants that cannot be breast-fed. Supplementation of the maternal diet with prebiotics functionally similar to HMO may also have potential health effects for both the mother and infant.

1.5.4.1 Ruminant milk oligosaccharides

Over the past decade, focus has been on the discovery, characterisation and profiling of ruminant oligosaccharides, especially bovine milk oligosaccharides (BMO) [260-264] as a possible source of milk oligosaccharides for infant nutrition. The number of oligosaccharides reported thus far in mature bovine milk (39) [264] and caprine milk (20) [265] is less than the 200 different structures reported in human milk [266]. Some of the predominant caprine milk oligosaccharides (CMO) and BMO, are structurally similar to those in HMO suggesting that they may have identical or similar functions as described for HMO (Table 1.2) [267].

Oligosaccharide enriched products obtained from caprine milk were shown to modulate the abundance of goblet cell specific genes in the HT29/MTX cell model [268], and to reduce the inflammation of the large intestine in a rat model of dextran sodium sulphate induced colitis [269]. The paucity of studies exploring the biological functions of ruminant milk oligosaccharides is attributable to the limited number of efficient methods of producing oligosaccharide enriched products.

Table 1.2 Caprine milk oligosaccharides described in the literature and oligosaccharides also reported in human* and/or bovine milk#. Concentration of caprine milk oligosaccharides (G), human milk oligosaccharides (H) and bovine milk oligosaccharides (B) reported.

Common name	m/z	Conc. (g/L)	Reference
α-2′-Fucosyl-lactose*	487.17	traces [G] 0.006–1.36 [H] n.r.[B]	[270-272]
α-3′-Galactosyl-lactose*#	503.16	0.03-0.05 [G] n.r.[H]; n.r.[B]	[265, 270, 273, 274]
β-6′-Galactosyl-lactose*#	503.16	0.04-0.016 [G] n.r.[H]; n.r.[B]	[270, 273, 274]
6'-*N*-Acetyl-glucosaminyl-lactose	544.19	0.02-0.04 [G]	[265, 275]
6'-Sialyl-lactose*#	632.21	0.05-0.07[G] 0.04-0.4[H] 0.005-0.006[B]	[261, 265, 276-278]
3'-Sialyl-lactose*#	632.21	0.03-0.05[G] 0.04-0.14[H] 0.04-0.05[B]	[261, 265, 276-278]
6'-Glycolyl-neuraminyl-lactose	648.20	0.04-0.06 [G]	[279]
6'-Sialyl-lactosamine#	673.23	traces [G] 0.0001[B]	[261, 276]
Di-*N*-acetyl-glucosaminyl-lactose	747.27	traces [G]	[265]
3'-Sialyl-6'galactosyl-lactose	794.26	traces [G]	[265]
6'-Sialyl-3'galactosyl-lactose	794.26	traces [G]	[265]
N-Glycolyl-neuraminyl-hexosyllactose	810.26	traces [G]	[265]
Disialyl-*N*-lactose#	923.30	0.001-0.005 [G] 0.001[B]	[261, 265]
Sialyl-*N*-glycolyl-neuraminyllactose	939.30	traces [G]	[265]
Di-*N*-glycolyl-neuraminyl-lactose	955.29	traces [G]	[265]
Lacto-*N*-hexaose*#	1071.38	0.001-0.005 [G] n.r.[H]; n.r.[B]	[265, 280, 281]

Note. n.r., concentration not reported.

The sialyloligosaccharides (N-glycolylneuraminyl-lactose, 3'-sialyl-lactose and 6'-sialyl-lactose) were shown to be the most prevalent oligosaccharides in caprine milk [279]. Over the last two decades, sialyloligosaccharides, such as 3'- and 6'-sialyl-lactose, have been shown to have a range of biological functions in humans. These acidic oligosaccharides increased the number of bifidobacteria in neonatal GIT [282] and promoted a GIT microbiota resistant to dextran sulfate sodium induced colitis in adult mice [283]. There are indications that they may reduce the severity of influenza virus infection and ulcers caused by *H. pylori* [284]. Other functions include increased immunity in infants, development of cerebral function and enhanced proliferation of commensal enteric bacteria [72, 237, 285, 286]. Sialyloligosaccharides represent 12 out of 23 predominant HMOs [271, 278], but only five out of six [261] and four out of seven predominant BMO and CMO, respectively [265]. Although there is a reduced number of sialyloligosaccharides in caprine milk compared with BMO, their concentrations are two or three times higher (500-900 mg/L) than in bovine milk (60 mg/L) [261] and bovine infant formulas (15–35 mg/L) [262]. The higher concentration of sialyloligosaccharides in caprine milk and their potential health effects (described in human milk [282]), indicate that CMO is a promising ingredient for promoting health in both infants and adults.

1.6 Conclusion and future perspectives

Today, non-communicable diseases are the biggest contributors to human morbidity and mortality. In the late 1980s, the "Developmental Origins of Health and Disease" hypothesis established the principle that the incidence of non-communicable adult diseases may be linked to *in utero* development. During the last decade, important evidence has shown that during pregnancy, maternal GIT microbiota or its products may be transferred to the foetus/neonate through the placenta or by breastfeeding. Further studies, however, are required to define the mechanisms by which the microbiota present in the mother's GIT

and/or genital tract may influence maternal physiology, and the development and maturation of the offspring with health consequences in later life.

Most human intervention studies evaluating the effects of perinatal administration of probiotics and prebiotics on pregnant women and to infants after birth focus primarily on the prevention of atopic dermatitis. Although these findings indicate some positive effects, there are also conflicting results dependent on the specific strains tested, the conditions of use, and the population studied. Administration of probiotics and/or prebiotics during the perinatal and postnatal period may be a potential prophylactic therapy for other modern life diseases and conditions, such as obesity and other metabolic diseases. There is an urgent need for long term human intervention studies to test this hypothesis, and to widen our knowledge of the interactions between maternal environment and maternal and foetal health outcomes.

There has been great interest in the use of ruminant milk oligosaccharides as functional ingredients for human health. Some enrichment methods have been developed using bovine whey to produce oligosaccharide enriched products which could be used for *in vitro* and *in vivo* studies. This enrichment methodology still needs to be tested on other milk types, such as caprine.

1.7 Hypotheses and Aims

The microbiota of the GIT are mainly acquired at birth and their rate, type and pattern are important factors for the overall health of the infant and may also have effects on the health status in later life. Bifidobacteria, for example, are health promoting bacteria that are differently present in breast-fed and in formula-fed babies. The microbial colonisation and development of the foetal and infant GIT occurs under the partial control of the maternal environment. The maternal environment is mainly influenced by the mother's diet and her GIT microbiota composition. Attempts to improve the maternal GIT microbiota by supplementation with synthetic oligosaccharides have been done successfully; however,

transfer of these benefits to the offspring remains to be proven. Human milk is unique in the amount of complex natural oligosaccharides that stimulate growth of health promoting bacteria as a core component of the GIT microbiota. Consumption of human milk is also able to improve immune function; prevent adhesion of pathogens to GIT epithelial tissues; increase absorption of minerals and improve glucose homeostasis. Among ruminant milk, caprine milk has the oligosaccharide profile and structural characteristics most similar to human milk and may thus be capable of improving the GIT microbiota colonisation and metabolic activity; enhance the development of the neonatal immune system and mucosal tissue maturation of the infant GIT.

The overall aim of this thesis was to explore (in mice) the effects of CMO on the dam's GIT microbiota, and milk composition, and subsequent effects on her offspring's GIT tissue development and maturation. The key hypotheses to be tested were:

Hypothesis 1: Bifidobacteria selected from the faeces of breast fed infants are able to ferment a caprine milk oligosaccharide enriched product (CMOP) and accelerate the development and maturation of the mono-associated mouse large intestine mucosa.

Hypothesis 2: Dietary CMOP directs the maternal large intestine microbiota toward one with increased fermentative capacity, leading to increased milk nutritive value.

Hypothesis 3: Dietary CMOP induced changes in the maternal large intestine microbiota and milk composition accelerates the development and maturation of the offspring's large intestine tissue and alters the GIT microbiota composition.

Hypothesis 4: The effects of maternal CMOP supplementation on the offspring's large intestine tissue and GIT microbiota composition are detected after 30 days consuming control diet.

The specific aims and major analytical approaches used in this dissertation for testing the stated hypotheses were:

1. Produce a CMOP from liquid whey. Process approximately 300 L of caprine whey to obtain a CMOP needed for the *in vitro* and *in vivo* studies described in Aims 2 to 5. The enrichment process was whey filtration, lactose hydrolysis, and adsorption on to carbon.

2. Obtain bifidobacteria from the faeces of breast fed human infants, determine which are capable of fermenting CMOP, and test the effects of the best strain on the morphology of the colonic mucosa of GF and conventionally raised mice, supplemented with dietary CMOP. Seventeen bifidobacteria strains isolated from the nappies of breast fed infants were identified by sequencing of the 16S rRNA gene. Random Amplification of Polimorphic DNA (RAPD) analysis was used to separate isolates on the basis of RAPD profiles, providing an indication of contrasting genotypes. The strains were cultured in medium with various carbohydrates including oligosaccharides, and the concentrations of SCFA in the medium were measured. Alterations in colonic crypt length and goblet cell numbers in response to CMOP feeding and bifidobacteria colonisation were assessed in transverse sections of the colon.

3. Analyse changes in the microbiota colonisation and fermentative capacity of the large intestine in lactating conventionally raised mice in response to dietary CMOP. Large intestine microbiota profiles were determined by 454 sequencing of the bacterial 16S rRNA gene. Large intestine SCFA concentrations were measured by gas chromatography equipped with a flame ionisation detector (GC-FID).

4. Assess changes in milk nutrient composition of lactating conventionally raised mice in response to CMOP induced changes in large intestine microbiota composition

and metabolism. Milk total protein, sugars, and fat concentrations were measured by Bradford, HPLC/ and LC-MS, Röse-Gottlieb method respectively.

5. Determine the effects of CMOP induced changes in the conventionally raised dam large intestine function on the development and maturation of the offspring's large intestine mucosa and microbiota. Alterations in the offspring's colonic crypt length and goblet cells numbers were assessed in transverse sections of large intestine tissue. Large intestine microbiota profiles were determined by 454 sequencing of the bacterial 16S rRNA gene. Large intestine SCFA concentrations were measured by GC-FID.

A flowchart showing the structure of the dissertation is presented in Figure 1.6.

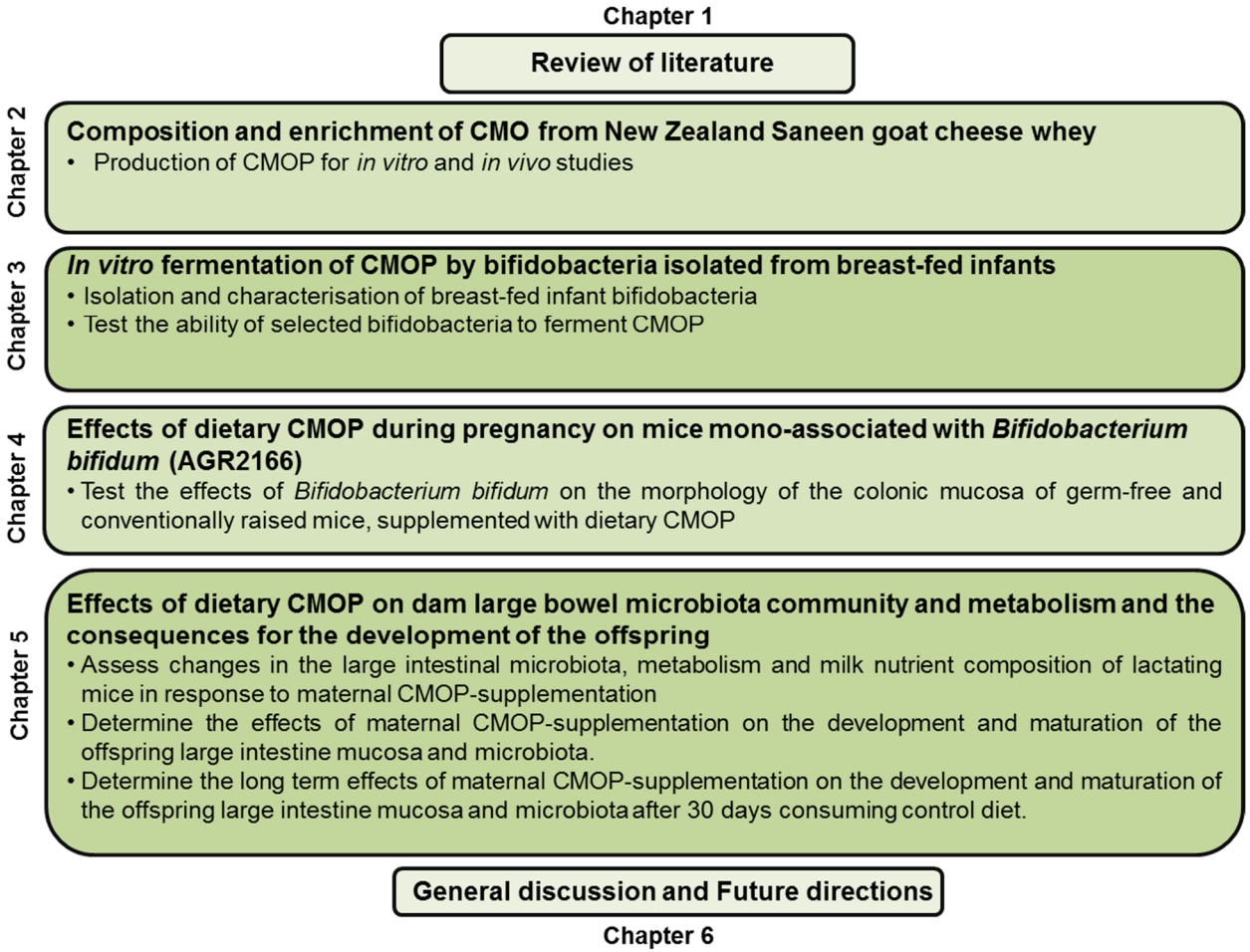


Figure 1.6 Structure of the dissertation. A flow chart describing the outline of the dissertation and its experimental Chapters. CMO, caprine milk oligosaccharides; CMOP, caprine milk oligosaccharide enriched product.

Chapter 2 Composition and enrichment of caprine milk oligosaccharides from caprine whey

The results presented in this chapter were published in the Journal of Food Composition and Analysis:

Thum, C., A. L. Cookson, W. C. McNabb, N. C. Roy. and D. E. Otter. Composition and enrichment of caprine milk oligosaccharides from New Zealand Saneen goat cheese whey. Journal of Food Composition and Analysis. 2015. **42**: 30-37.

Chapter 2

2.1 Introduction

Caprine milk production is a dynamic and growing industry that is fundamental to the wellbeing of millions of people worldwide, and an important part of the economy in many countries [287, 288]. In New Zealand, the dairy caprine co-operative has 50 exclusive breeders with a total herd size of 30,000 animals, predominantly Saanen. Most of the milk (20 million litres per year) is used to manufacture infant formula which is exported to around 20 countries [289]. The increasing demand for caprine milk for infant nutrition is evidenced by widespread reports of the use of raw caprine milk and homemade formulas for infants [287, 290, 291].

Infant formulas are used when there is not enough breast milk, or breast feeding is not possible. Caprine milk provides an alternative base for the production of infant formulas, as it's known for its relatively lower allergenic burden, easier digestion and physiological benefits, compared to cow milk [269, 292-294]. Differences in milk composition have been linked to those physiological effects. The amount of lactose in bovine and caprine milk is similar, but Alpha-s1 casein (only present in ruminant milk) is found in lower concentrations in caprine compared to bovine milk [293, 294]. Caprine milk contains higher concentrations of nucleotides, polyamines, and some of the essential amino acids [295, 296]. Caprine, bovine and human milk also contain non-nutritional components, such as oligosaccharides, in different concentrations and structures that possibly play a role in the different effects seen from formula feeding.

Oligosaccharides (non-digestive sugars, known for their ability to stimulate growth of health promoting bacteria in the GIT), are highly concentrated in human milk (5 to 23 g/L), and structurally diverse with more than 200 different oligosaccharides reported [141]. Consumption of HMO are known to improve immune function [297] and prevent adhesion of pathogens to intestinal epithelial tissues [298]. Lower rates of diarrhoea, respiratory diseases, allergies and

inflammatory diseases have been reported, among breast fed infants, compared with formula fed infants [299], which may be related to the consumption of HMO [300].

The oligosaccharide composition of caprine and bovine milk has been intensely studied in the past decade [260-264, 301]. The number of oligosaccharides reported thus far in bovine milk (up to 40) [264, 302], bovine colostrum (over 50) [272] and caprine milk (40) [301] is less than the 200 different structures reported in human milk [266, 272]. Some of the predominant CMO and BMO are structurally similar to those in HMO (Table 1.2) suggesting that, to some extent, they may have identical or similar functions as described for HMO [267].

Few studies have explored the physiological effects of CMO. Oligosaccharide enriched products obtained from caprine milk, for example, have been shown to modulate the abundance of goblet cell-specific genes in the HT29/MTX cell model [268] and to reduce intestinal inflammation in a rat model of dextran sodium sulphate induced colitis [269]. More studies are needed to determine other biological functions of CMO, especially the potential effects of these non-nutritional components on the development of the GIT.

2.2 Hypothesis and Aims

The first hypothesis of the research presented in this chapter was that New Zealand Saanen caprine milk and whey have a similar oligosaccharides profile to that previously identified in studies with other caprine breeds. The second hypothesis is that a method based on bovine whey lactose hydrolysis, centrifugation and porous graphitic carbon adsorption chromatography produce a CMOP on a scale sufficient for subsequent *in vitro* and *in vivo* experimentation.

The first aim of the present study was to investigate the oligosaccharide composition of New Zealand Saanen caprine milk and whey. The second aim was to develop a method to provide an enriched, free of protein and partially characterised oligosaccharide fraction from New Zealand Saanen caprine milk.

2.3 Materials and methods

2.3.1 Milk and whey origin and chemical characterisation

An initial characterisation of various caprine milk streams suggested that whey would be the most appropriate starting material. The method described here uses caprine cheese whey as the raw material, together with combinations of enrichment strategies previously used to purify and/or structurally identify CMO and BMO [303-305], to purify and characterise the composition of a potential New Zealand Saneen caprine milk by-product.

New Zealand Saanen caprine colostrum, pasteurised milk and whey (from camembert cheese making) were obtained from Over the Moon Dairy Company Limited (Putaruru, New Zealand). Colostrum, milk and whey samples were refrigerated during transportation and stored frozen at -20°C if not processed immediately. Aliquots of each sample were analysed for dry matter, lipid and carbohydrate composition as detailed below.

2.3.2 Proximate composition

Colostrum, whey and milk composition, except sugars, were analysed by the Nutrition Laboratory Institute of Food, Nutrition and Human Health, Massey University (Palmerston North, New Zealand).

For dry matter analysis, samples were dried in oven at 135ºC for 2 h (Association of Official Analytical Chemists (AOAC International) method 930.15 [306]). Crude protein was analysed using the Dumas method, AOAC International 968.06 [307]. In the Dumas method, protein N_2 is freed by pyrolysis and subsequent combustions, is swept up by CO_2 and converted to equivalent protein by numerical factor. Crude fat of colostrum, whey and milk samples were determined using the Mojonnier method, AOAC International 954.02 [308]. In the Mojonnier method, ether and alcohol are used to extract the fat. The extract is decanted into a dry weighing dish and the

ether is evaporated. The extracted fat is then dried in an air oven at 100ºC, cooled in a desiccator, to a constant weight and expressed as percentage of fat by weight.

2.3.3 High performance liquid chromatography

The carbohydrate composition of colostrum, milk and whey samples was determined by high performance liquid chromatography (HPLC) for galactose, glucose and lactose, and liquid chromatography–mass spectrometry (LC-MS) for oligosaccharides.

Samples of caprine colostrum, milk, whey were ultra-filtered using a 10 KDa membrane in an Amicon ultrafiltration cell (Model 8200, Millipore, Danvers, MA USA) to remove proteins prior to analysis. Freeze dried oligosaccharide enriched product was reconstituted with reverse osmosis water to a final concentration of 5 g/L. The samples were analysed for lactose, glucose, galactose and GOS concentration using a Shimadzu LC10A HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand) fitted with a Bio-Rad Aminex HPX 87H HPLC column (maintained at 45°C) and a Shimadzu refractive index detector, RID10A. The mobile phase was sulphuric acid (5 mM) using an isocratic elution with a flow rate of 0.8 mL/min^{-} Injection volumes were 50 µL with a run time of 15 min. Sugars were quantified using calibration curves prepared for lactose, D-glucose, D-galactose (BDH, UK), and GOS (Yakult, Japan) (0.1 to 2.5 g/L.

HPLC is an important tool to identify and quantify carbohydrates in food samples. Selection of the optimal HPLC approach depends on the sample matrix, carbohydrate concentration, selectivity and sensitivity required [309]. The Aminex HPX 87H column is optimised for analysing monosaccharides and also provides class separation of di-, tri-, and tetrasaccharides. The refractive index detector was used to the direct determination and quantification of sugars present in the samples. The refractive index detector works on the principle of differential refractometer and is typically of the deflection type. The flow cell is divides into a reference liquid compartment and a sample liquid compartment. A light beam passing between these two compartments is

deflected in proportion to the difference in refractive index between the reference and sample liquids. The deflection is measured by the displacement on a light-receiving element [309].

2.3.4 Liquid chromatography–mass spectrometry

Structural analysis of complex mixtures of oligosaccharides using tandem mass spectrometry is often complicated by the presence of a multitude of structural isomers. Detailed structural analysis is, therefore, often achieved by combining oligosaccharide separation by HPLC with online electrospray ionization and mass spectrometric detection. A very popular and promising method for analysis of oligosaccharides is graphitized carbon LC-MS. Oligosaccharides and GOS were characterised and quantified by LC-MS (Q-Exactive, Thermo Fisher Scientific, Waltham, MA, USA). 20 μL samples were prepared as for HPLC analysis, and were separated on a Thermo Hypercarb column (100 x 2.1 mm, 5 μm particle size, Thermo Fisher Scientific, NZ) at a flow rate of 0.4 mL/min with a gradient programmed as follows: initial 1% B (0.1% formic acid in LiChroSolv acetonitrile, Merck, Palmerston North, NZ) held for 0.5 min, to 10% B after 1 min, to 16% B at 6 min, to 20% B at 12 min, to 30% B at 16 min, to 90% B at 18 min, held for 2 min, and then returned to initial conditions (1% B) for a 5 min equilibration. Water was used as mobile phase A [310].

Oligosaccharide characterisation and quantification was performed on a Thermo Scientific LTQ XL-Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionisation in negative mode. Data were collected in profile data acquisition mode over from 300 to 2000 mass to charge ratio (*m/z*) and processed using the Xcalibur software package provided by the manufacturer. Calibration curves were; 4-galactosyllactose (Glentham Life Sciences, UK), 3'- and 6'-sialyl-lactose milk oligosaccharide; α-3'-and α-2'-fucosyllactose, lacto-N-hexaose, disialyllactose, lactose 1-phosphate (Carbosynth, UK) and GOS (kindly donated by Yakult, Japan) (0.01-0.5 g/L). While it was recognised that the various oligosaccharides will have different ionisation efficiencies during MS analysis, the lack of purified oligosaccharide calibration standards meant that for this study, oligosaccharide quantification was estimated

using the response curves of the purified 3- and 6-sialyl-lactose where no oligosaccharide standards were available.

2.3.5 Caprine whey oligosaccharide enrichment process

Ten litres of caprine whey were processed in eight batches (approximately 1200 mL per batch). Each batch was subject to lactose hydrolysis, centrifugation, porous graphitic carbon adsorption chromatography (in 4 x 300 mL aliquots per batch), solvent evaporation and freeze drying as described below (Figure 2.1).

2.3.5.1 Centrifugation and ultrafiltration

Whey samples (pH 4.5 to 5.0) were centrifuged in a Sorvall RC5 plus (Thermo Fisher, Langenselbold, Germany) at 25,000 x *g* for 10 min at 4°C, and then ultra-filtered using a 6.7 m^2 10 kDa cut off spiral wound ultrafiltration membrane (Desal model PW1812C, GE Technology). The filtration operating conditions were: temperature, 55°C; pH, 5.5; and pressure inlet and outlet pressures, 1.8 and 1.4 bar, respectively, with an average flow rate of 80 mL/min The retentate containing whey protein concentrate was discarded and permeate containing oligosaccharides was hydrolysed as described below.

2.3.5.2 Lactose hydrolysis

The whey permeate was incubated with β-galactosidase (*Aspergillus oryzae*, Sigma) at 3 U/mL, 40°C and pH 4.5, for 24 h to hydrolyse lactose to glucose and galactose. After incubation, the whey pH was adjusted to 7.5 with 5M sodium hydroxide to stop the hydrolysis, then refrigerated for a minimum of 12 h before further processing. To remove any particulate material, the whey was then centrifuged at 25,000 x *g* (Sorvall RC6 plus, Thermo Scientific) for 15 min at 4°C, prior to porous graphitic carbon adsorption chromatography.

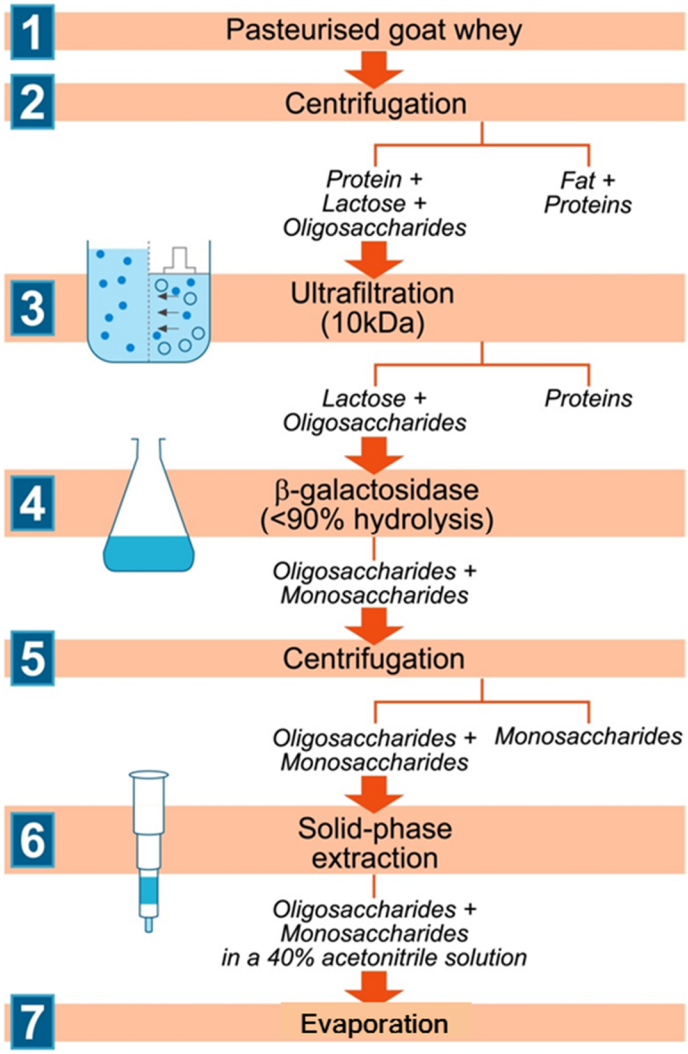


Figure 2.1 Diagram of the experimental method, described in seven main steps, to produce a caprine milk product enriched with oligosaccharide. (1) Pasteurised caprine whey was (2) centrifuged to reduce the concentrations of fat and proteins in the initial whey. (3) Ultrafiltration step was used to remove the remaining protein and (4) β-galactosidase to hydrolyse the lactose present in the whey permeate. (5) Centrifugation and the (6) solid phase extraction removed the majority of the monosaccharides and remaining lactose from the hydrolysed whey. (7) Acetonitrile, used to elute the oligosaccharides from the solid phase extraction, was removed by evaporation.

2.3.5.3 Porous graphitic carbon adsorption chromatography

The oligosaccharide solution was put into a glass column (127 mm diameter, glass fritted disc, porosity 40 to 60 μm) containing 300 g of porous graphitic carbon media (30-40 μm Hypercarb, Thermo Fisher) using a strategy similar to that of Barile *et al* (2009) for bovine whey oligosaccharide purification (on a larger scale and using different carbon media). One column volume (300 mL) of hydrolysed whey was loaded onto the column. Oligosaccharides bound to the porous graphitic carbon media, while the majority of the remaining glucose, galactose and non-hydrolysed lactose (65%) were washed out using three column volumes of distilled water. The bound oligosaccharides together with the remaining monosaccharides and lactose were then eluted with one column volume of 40% (v/v) acetonitrile solution. The carbon media was then re-equilibrated with water and reused up to twenty times. The adsorption and elution conditions were initially optimised with different solutions of acetonitrile (2 or 5% adsorption; 40 or 50% elution) and ethanol (20%, 40%, 50%) and different elution volumes (0.5, 1.0, 2.0 column volumes) (Table 2.1).

2.3.5.4 Solvent evaporation and freeze drying

Acetonitrile was removed from the oligosaccharide solution by rotary evaporation (1.2 L/h (Buchi Rotavapor R-114, Switzerland) under vacuum (Buchi Vacuum pump V-700, Switzerland) in a 55°C water bath (Buchi Waterbath B-480, Switzerland). Evaporation was stopped when the oligosaccharides solution reached approximately 50% of the original volume. As the recovered acetonitrile solution contained both acetonitrile and water it was discarded. The oligosaccharide solution was then frozen and freeze dried to get the final product.

Table 2.1 Optimisation of adsorption and elution conditions used for porous graphitic carbon chromatography. The adsorption and elution conditions were optimised with different solutions of aqueous acetonitrile (2 or 5% adsorption; 40 or 50% elution) and ethanol (20%, 40%, 50%), and different elution volumes (0.5, 1.0, 2.0 column volumes (V)). Recovery of oligosaccharides and contaminants are expressed as percentage of the initial concentration of these sugars present in the whey.

Solution	Adsorption	Elution	Recovery (%)	
			Oligosaccharides	Contaminants (glucose, galactose and lactose)
Ethanol	-	1 V, 20%	42	13
	-	1 V, 40%	70	24
	-	1V, 50%	75	25
	-	0.5 V, 50%	59	18
	-	2 V, 50%	80	36
Acetonitrile	1V, 2%	1V, 40%	70	10
	1V, 5%	1V, 40%	70	12
	-	1V, 40%	80	5
	-	1V, 50%	87	16
	-	0.5V, 40%	55	5
	-	2V, 40%	80	16

2.4 Results

2.4.1 Source of caprine milk oligosaccharides

In the present study, camembert cheese whey was produced by adding *Lactococcus lactis* subsp *cremoris*, *Lactococcus lactis* subsp *lactis* and *Streptococcus thermophilus* to caprine milk as starter cultures, along with the fungus *Geotrichum candidum*. The gross composition of New Zealand Saanen caprine colostrum, milk and whey are shown in Table 2.2.

The starter culture may have hydrolysed part of the lactose (24%) present in the milk, and as a result, increased the concentration of glucose (13-fold) and galactose (135-fold) present in the whey [165]. The higher concentration of galactose compared to glucose may be due to preferences in glucose consumption by the starter culture. Although, *L. lactis* and *S. thermophilus* are able to synthesise GOS through the enzyme β-galactosidase [311, 312], no GOS was detected in the starting whey.

2.4.2 Whey processing and solid phase extraction

Initial centrifugation and ultrafiltration steps (to decrease the fat and protein concentration of the whey that interfered with the solid phase extraction stage), removed >98% of initial fat and protein content (Table 2.2). The β-galactosidase successfully converted 92% of the lactose to monosaccharides, reducing the lactose level from 25.6 g/L in the whey permeate to 0.9 g/L. However, the level of lactose was still greater compared to the oligosaccharide levels (0.16 g/L), thus the need for further processing. Part of the lactose, glucose and galactose was bound to, and eluted from, the porous graphitised carbon under the adsorption/desorption conditions used to bind and elute the oligosaccharides, and thus could not be separated from the oligosaccharides during this step.

No GOS was identified in the caprine colostrum, milk or whey samples based on LC-MS characterisation of typical GOS masses commonly detected by negative ion electrospray MS

Table 2.2 Dry matter, lipid, protein and carbohydrate composition of caprine colostrum, milk and whey. Galacto-oligosaccharide concentration is based on the sum of the two ion intensities, *m/z* 665 and *m/z* 827. Oligosaccharide concentrations are based on the sum of ion intensities, m/z 503, m/z 632, m/z 648, and m/z 923 (Table 1.2). Values are expressed in grams per liter, and are the average of single analyses of eight different batches.

Sample	pH	Average concentration (g/L)							
		Dry matter	Lipid	Protein	Lactose	Glucose	Galactose	GOS	Oligosaccharides
Colostrum	6.2	175	7	107	58	0.23	0.0025	0	0.32
Milk	6.5	115	36	30	47	0.15	0.0015	0	0.26
Whey	4.7	58	12	7	36	2	0.2	0	0.20
Ultrafiltration permeate	4.2	29	<0.1	0.1	25	2	0.3	0	0.20
Permeate hydrolysed and centrifuged	7.5	28	<0.1	<0.1	3	14	11	0.09	0.18
Solid phase extracted final product	-	2	<0.1	<0.1	0.9	0.5	0.4	0.08	0.16

(ions of 665, 827 and 989 *m/z*) [313, 314]. Low levels of GOS were detected by LC-MS in the hydrolysed permeate after 24 h of β-galactosidase incubation and final product (Table 2.2). An additional potential GOS ion at *m/z* 503 may have also been produced by β-galactosidase activity; however, it was not possible to distinguish this ion from one already present in the whey (retention time of 4.09 min, *m/z* 503.16). Optimisation of hydrolysis time and β-galactosidase concentration was required to obtain a balance between lactose hydrolysis and GOS production. Tests showed that if lactose hydrolysis was carried out for longer, or with higher concentrations of enzyme, GOS was produced at a higher rate than lactose was hydrolysed. Whey incubated with β-galactosidase for 36 h, for example, increased the lactose hydrolysis by 5% but also increased the production of GOS by 50%.

β-galactosidase activity was stopped by raising the pH of the hydrolysed permeate to 7.5 and decreasing the temperature to 4°C. During refrigeration, a portion of the galactose (7.5%) produced by lactose hydrolysis was precipitated, and this was removed by centrifugation. After loading the β-galactosidase treated sample onto the porous graphitised carbon, the column was washed with three column volumes of deionised water. This partially removed the carbohydrate contaminants (glucose, galactose, GOS and lactose), together with the salts and residual traces of protein and lipids (data not shown) prior to elution with 40% acetonitrile. A total of 2.5% of lactose and 80% of oligosaccharides were recovered in 9.6 L of 40% acetonitrile solution (Table 2.3). From 8 batches (approximately 1200 mL per batch) of whey, 16 g of product were generated of which around 8% were oligosaccharides, 44% monosaccharides, 44% lactose and 4% GOS. As the starting material (Saneen caprine whey) contained only 0.34 % oligosaccharides, the enrichment process has resulted in a 24-fold increase in the relative levels of oligosaccharides with an 80% recovery.

Table 2.3 Recovery rate of lipid, protein and carbohydrate during whey ultrafiltration, hydrolysis and solid phase extraction. Concentrations adjusted on the basis of stated volume.

Sample	Volume (L)	Recovery (%) of lipid, protein and carbohydrate through whey processing			
		Lipid	Protein	Lactose	Oligosaccharides
Whey	9.6	100	100	100	100
Ultrafiltration permeate	8.5	<0.1	1	62	85
Permeate hydrolysed and centrifuged	8.2	<0.1	<0.1	7	76
Solid phase extracted final product	9.6	<0.1	<0.1	2.5	80

2.4.3 Liquid chromatography–mass spectrometry analysis

Typical LC-MS profiles of the ions corresponding to the major oligosaccharides observed in Saanen caprine whey and in the final enriched oligosaccharide product are show in Figure 2.2 A and B respectively. The putative identification and quantification of oligosaccharides in the available standards of oligosaccharides reported in caprine [265, 270, 276] and bovine [272] milk (samples was performed using commercially). To illustrate the profile of the main oligosaccharides present in the enriched product, a typical MS spectrum is provided in Figure 2.3. In this spectrum, the most abundant ions (*m/z* 503, *m/z* 632 and *m/z* 648) corresponded to the masses of the oligosaccharides galactosyl-lactose (α-3'- or β-6'-), sialyl-lactose (3'- or 6'-) and N-glycolylneuraminyl-lactose. Adduct ions, consistent with the covalent linking of the neutral sugar galactosyl-lactose (*m/z* 503) to a molecule of formic acid (46 *m/z*), at 549 *m/z* were also observed. It is interesting to note that the formic acid adduct is only detected for the neutral sugars, not the acidic ones. Traces of other possible CMO, based on previously reported masses described in Table 1.2, were also observed in the colostrum, milk, whey and the final purified product. Another feature of the MS profile were ions consistent with lactose 1-phosphate (*m/z* 421), another compound produced during lactose and galactose metabolism [315]. Lactose 1-phosphate was detected in milk (0.01 ± 0.002 g/L), whey (0.007 ± 0.001 g/L) and in the purified product (0.006 ± 0.002 g/L). Lactose 1-phosphate was first detected in bovine milk [316] and, recently reported in caprine milk [301].

Oligosaccharides concentrations were measure in 4 samples of caprine colostrum and milk and 8 samples of whey (Table 2.4). Caprine colostrum had higher concentration of oligosaccharides (ranging between 0.20 to 0.40 g/L (sum of the four most concentrated oligosaccharides (3' galactosyl-lactose (*m/z* 503), 3'-sialyl-lactose (*m/z* 632), 6'-sialyl-lactose (*m/z* 632), *N*-glycolylneuraminyl-lactose (*m/z* 648) and Disialyl-*N*-lactose (*m/z* 923)) compared to mature milk (ranging between 0.19 to 0.31 g/L). Caprine whey had lower concentrations of oligosaccharides (ranging between 0.11 to 0.27 g/L) compared to caprine milk. Oligosaccharide concentrations

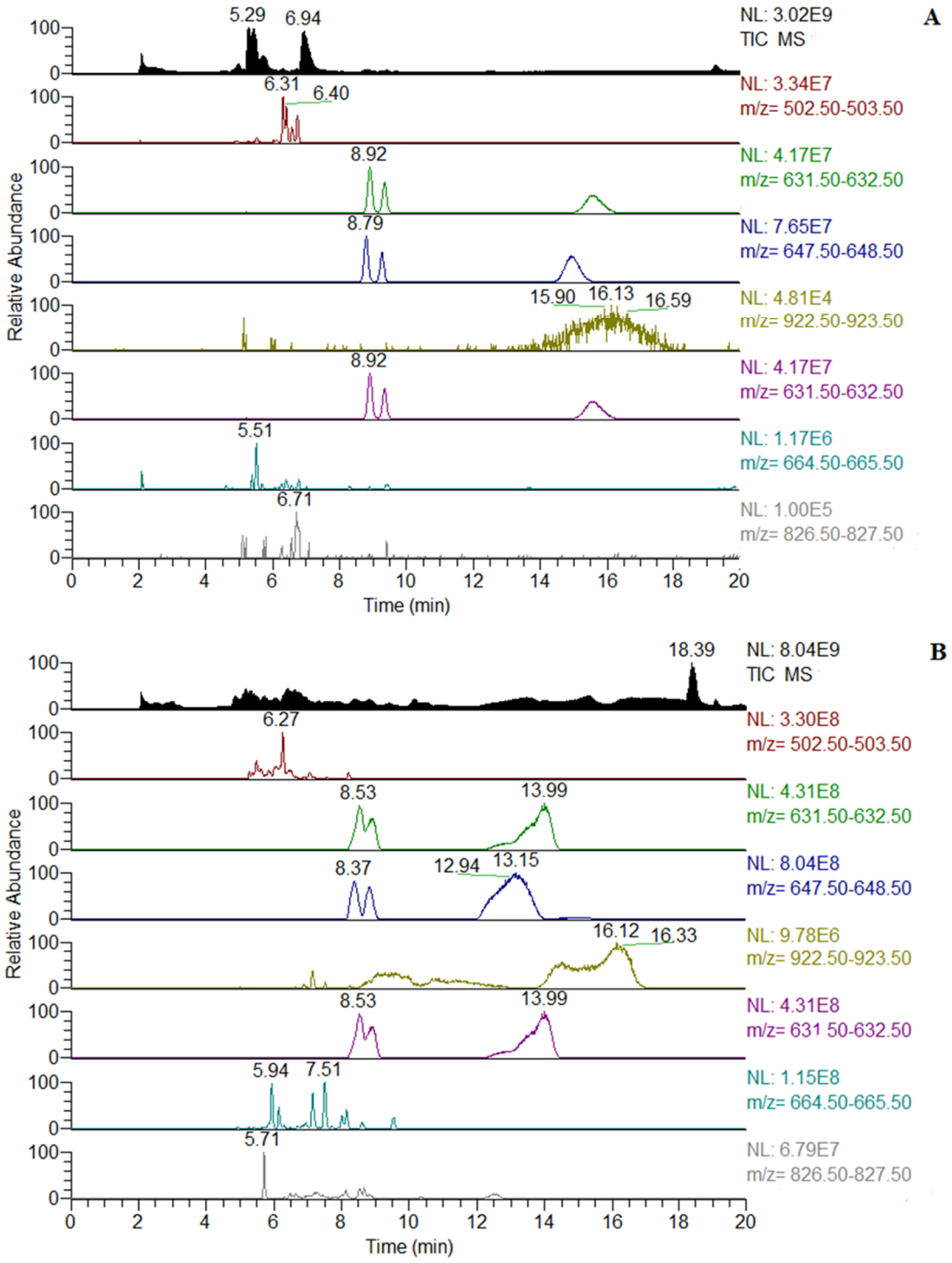
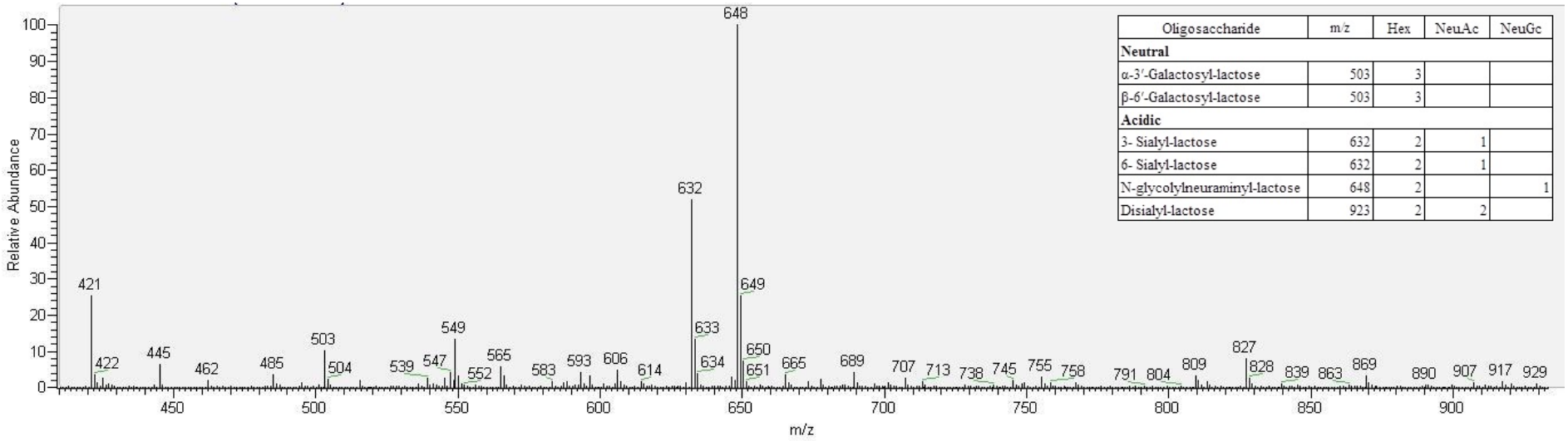


Figure 2.2 Liquid chromatography-mass spectrometry extracted ion chromatograms of (A) caprine whey and (B) purified product, showing the ions with *m/z* 503.1 (3-galactosyl-lactose); *m/z* 632.3 (3'-sialyl-lactose and 6'-sialyl-lactose); *m/z* 648.3 (*N*-glycolylneuraminyl-hexosyl-lactose); 923.3 (disialyl-lactose); *m/z* 665.2 (galacto-oligosaccharide) and *m/z* 827.3 (galacto-oligosaccharide).



Oligosaccharide	m/z	Hex	NeuAc	NeuGc
Neutral				
α-3′-Galactosyl-lactose	503	3		
β-6′-Galactosyl-lactose	503	3		
Acidic				
3- Sialyl-lactose	632	2	1	
6- Sialyl-lactose	632	2	1	
N-glycolylneuraminyl-lactose	648	2		1
Disialyl-lactose	923	2	2	

Figure 2.3 Mass spectrum of the product collected with a 40% acetonitrile solution from solid phase medium. The spectrum, showing the main chromatogram peaks between the retention time 5-12 minutes, was recorded using an LTQ linear ion trap mass spectrometer with electrospray ionisation in negative mode.

Table 2.4 Oligosaccharides concentration (g/L) in caprine colostrum, milk, whey, hydrolysed whey and final product using the liquid chromatography-mass spectrometry method and standards. Caprine milk oligosaccharides are represented by *m/z* 503, *m/z* 632, *m/z* 648, and *m/z* 923; *m/z* 487, *m/z* 1071 and GOS by *m/z* 665 and *m/z* 827. Due to the lack of commercial standard, N-glycolylneuraminyl lactose (*m/z* 648) concentrations were estimated based on 3'-sialyllactose (*m/z* 632) standard.

Sample	***n***	***m/z 503***	***m/z 632 (3'-SL)***	***m/z 632 (6'-SL)***	***m/z 648***	***m/z 923***	***m/z 487 (2'-FL)***	***m/z 1071***	***m/z 665***	***m/z 827***
Colostrum	4	0.08 ± 0.02	0.05 ± 0.02	0.07 ± 0.02	0.04 ± 0.02	0.05 ± 0.004	traces	0.03 ± 0.02	-	-
Milk	4	0.08 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	0.01 ± 0.008	0.002 ± 0.001	traces	-	-
Whey	8	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.002 ± 0.001	traces	traces	-	-
Hydrolysed whey	8	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.04	0.001 ± 0.001	traces	traces	0.04 ± 0.01	0.05 ± 0.01
Final product	8	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.05 ± 0.03	traces	traces	traces	0.05 ± 0.01	0.03 ± 0.01

decreased during processing and the oligosaccharide concentration found in the caprine enriched producted ranged between 0.07 to 0.23 g/L (Table 2.4).

2.5 Discussion

The goal of this study was to investigate the composition of milk and whey from the Saanen caprine breed in New Zealand, for the hypothesis; *New Zealand Saanen caprine milk and whey have a similar oligosaccharides profile to that previously identified in studies with other caprine breeds.* The data shown here is in accordance with the ions and concentrations observed in other caprine breeds [265, 317-320] (Table 1.2), from around the world. The second hypothesis, *The method of whey lactose hydrolysis, centrifugation and porous graphitic carbon adsorption chromatography will produce an CMOP on a scale sufficient for subsequent in vitro and in vivo experimentation,* was confirmed by the production of a CMOP containing 8% oligosaccharides, 44% monosaccharides, 44% lactose and 4% GOS.

2.5.1 Caprine whey processing

Caprine whey was chosen as the starting material for further enrichment of CMO as most of the fat and protein had already been removed during cheese making, simplifying further oligosaccharide enrichment. After initial centrifugation and ultrafiltration steps, (to decrease the fat and protein concentration of the whey), β-galactosidase was used to hydrolyse the lactose to avoid lactose binding to the porous graphitised carbon under the adsorption/desorption conditions used to bind and elute the oligosaccharides. However, the conditions utilised in this study were only sufficient to hydrolase 92% of the lactose to the monosaccharides, glucose and galactose. The inhibitory effect of higher concentrations of galactose in batch systems on lactose hydrolysis have been reported previously [301, 302]. Higher conversion rates may be achieved by continuous systems using immobilised β-galactosidase.

After loading the β-galactosidase treated sample onto the porous graphitised carbon, the column was washed with three column volumes of deionised water. This partially removed the carbohydrate contaminants (glucose, galactose, GOS and lactose) together with the salts and residual traces of protein and lipids prior to elution with 40% acetonitrile. A final product containing 8% oligosaccharides, 44% monosaccharides, 44% lactose and 4% GOS was obtained. Although acetonitrile is a solvent used in pharmaceutical preparations and it should be avoided in food additives [321], it is expected that complete acetonitrile removal was achieved by evaporation and freeze drying.

2.5.2 Oligosaccharide characterisation and quantification

A recent study has identified 40 different oligosaccharide structures in caprine milk and described the relative abundance of both acidic and neutral oligosaccharides using ultra-performance liquid chromatography [301]. The results of this current study use the relative intensities (peak areas) of extracted mass peaks from the mass spectrometric analysis for the quantification of CMO compared against the specific standards. While it is recognised that the intensity of each ion is dependent on the ability of that particular molecule to ionise in solution, approximate amounts of each putative oligosaccharide were estimated based on the appropriate calibration curves. The seasonality of milk yield, breed and degree of ionisation of the different oligosaccharides could be limitations of the current method and be responsible for variations in CMO concentrations and detection when compared to other studies. Although 40 different oligosaccharide structures have been identified in caprine milk by a recent study [301], the present study has identified and quantified only the most abundant oligosaccharides.

Martinez-Ferez *et al*. [265] compared the oligosaccharide concentrations among caprine, ovine, bovine and human milk and demonstrated that the CMO concentration was (0.25 to 0.30 g/L ten times higher than BMO (0.03 to 0.06 g/L) and ovine oligosaccharides (0.02 to 0.04 g/L), but less concentrated than HMO (5 to 10 g/L). Our results are similar with CMO

concentrations previously described, with the sum of the four most concentrated oligosaccharides (3' galactosyl-lactose (*m/z* 503), 3'-sialyl-lactose (*m/z* 632), 6'-sialyl-lactose (*m/z* 632), *N*-glycolylneuraminyl-lactose (*m/z* 648) and Disialyl-*N*-lactose (*m/z* 923)) in mature caprine milk from the Saanen breed in New Zealand, ranging between 0.19 to 0.31 g/L. The variation of oligosaccharide concentrations reported here and by other authors [279, 304] may be due variations of animal breed, feed, lactation stage and/or method of quantification. The approach described here (solid phase extraction with graphitised carbon and the LC-MS), however, could be used as a relatively rapid method to identify whey sources richest in oligosaccharides.

2.5.3 Sialyloligosaccharides prevalence and health effects

The sialyloligosaccharides (*N*-glycolylneuraminyl-lactose (*m/z* 648), 3'-sialyl-lactose (*m/z* 632) and 6'-sialyl-lactose (*m/z* 632)) are oligosaccharides highly prevalent in New Zealand Saanen caprine colostrum, milk and whey, although there was compositional variation between individual samples (Table 2.4). Over the last two decades, sialyloligosaccharides have been demonstrated to have a range of biological functions in humans. For example, 3'-sialyl-lactose and 6'-sialyl-lactose have been shown to increase the *in vitro* adhesion of bifidobacteria to intestinal cells [322] and promote, an intestinal microbiota resistant to DSS induced colitis in adult mice [283]. There are also indications that sialyoligosaccharides may reduce the severity of influenza virus infection and ulcers caused by *Helicobacter pylori* [284]. Other functions include increased immunity in infants, development of cerebral function, and enhanced proliferation of commensal enteric bacteria [72, 237, 285, 286].

The sialyloligosaccharide *N*-glycolylneuraminyl-lactose, however, contains the monosaccharide Neu5Gc, found in most mammalian milk (e.g. in the oligosaccharides 3'-sialyl-lactose and 6'-sialyl-lactose present in caprine and bovine milk), but not in human milk which contains Neu5Ac instead. Due to the fact that Neu5Gc differs from the human Neu5Ac, by only one oxygen atom, after the consumption of these oligosaccharides, human

cells may take up the Neu5Gc. The immune system does recognise this molecule as foreign, and this may be associated with increasing risk of many diseases, including carcinomas, atherosclerosis, and type-2 diabetes. Although not proven, caution is suggested when using oligosaccharides containing Neu5Gc in a human diet [323].

Sialyloligosaccharides represent 12 out of 23 predominant oligosaccharides found in human milk [271, 278], but only five out of six [261] and four out of seven predominant oligosaccharides described for bovine and caprine milk, respectively [279]. Although there is an equivalent percentage of sialyloligosaccharides in caprine (95%) and bovine milk (91%) compared to neutral oligosaccharides [301], their concentrations are two or three times higher in caprine milk (120-200 mg/L) than in bovine milk (60 mg/L) [261] and bovine infant formulas (15–35 mg/L) [262]. The presence of sialyloligosaccharides in caprine milk and their potential health effects in human milk [282] indicate that CMO may be used in the healthcare and food sectors as ingredients to promote health in both infants and adults.

2.6 Conclusions

The present study is the first to report New Zealand Saanen caprine colostrum, milk and whey composition. It also describes a multi-step approach to process caprine whey containing 0.3% oligosaccharides and produce an CMOP with a final CMO content of 8% (w/w) and with an oligosaccharide profile similar to the initial caprine milk. Using this method, eight batches of approximately 1.2 L of caprine cheese whey produced approximately 19 g of product containing 1.6 g CMO for use in *in vivo* experiments. The major contaminants of the enrichment process were lactose, glucose and galactose. The method recovered approximately 80% of the original oligosaccharides present in the cheese whey starting material. These results suggest that caprine whey from camembert cheese manufacture is a rich source of oligosaccharides and that analysis of the *m/z* data suggested that there are common oligosaccharides between New Zealand Saanen caprine and human milk. While New Zealand Saanen caprine milk has a lower oligosaccharides concentration when

compared to the published data from other CMO studies, the levels reported here are higher than those reported in bovine studies. Six out of seven predominant oligosaccharides, previously described for caprine milk, were putatively identified and quantified. The high concentrations of sialyloligosaccharides found in Saanen caprine milk in New Zealand provide a useful source for the *in vitro* and *in vivo* experimentation of their modes-of-action and to further determine the potential health benefits. Future studies are needed to confirm and compare the oligosaccharides concentrations for different caprine breeds, stage of lactation and diet.

Chapter 3 *In vitro* fermentation of caprine milk oligosaccharides by bifidobacteria isolated from breast-fed infants

The results presented in this chapter have been formatted as a paper which is in review with Gut Microbes Journal:

Thum, C., A. L. Cookson, D. E. Otter, W. C. McNabb and N. C. Roy (In review). *In Vitro* Fermentation of Caprine Milk Oligosaccharides by Bifidobacteria Isolated from Breast-Fed Infants.

Chapter 3

3.1 Introduction

Evidence suggests that the microbial community of the human gastrointestinal tract (GIT) has a core function in maintaining host health by preventing the colonisation of pathogens [324], degrading dietary compounds, producing metabolites able to be utilised by the host (e.g. SCFA) [325] and maintaining mucosal immunity. [326] Particularly important in early life, the composition of the GIT microbiota influences the development and maturation of the foetal/neonatal GIT and consequently the overall health of the infant. During the perinatal period, the infant GIT is colonised by a relatively simple microbial community, initially derived from vaginal microbiota and maternal faeces. [327] The type of feeding (breast versus formula feeding), local environment, and antibiotic treatment may also play an important role in determining and maintaining the microbiota composition of the infant GIT. [107, 124]

The GIT microbiota of breast fed infants has long been thought to be dominated by bifidobacteria (compared to that of adults and formula fed infants). However, a review of studies from the last 20 years reported only minor differences in the levels of bifidobacteria present in formula fed infant faeces when compared breast fed infants faeces [328]. In contrast, some studies have demonstrated a specific diversity of bifidobacterial species present in formula fed infants that is more like adult diversity [329, 330]. Moreover, certain microbial families (such as Clostridiaceae/Clostridiales, Bacteroidaceae, and Enterobacteriaceae), are more often found in formula fed, than breast fed infants, supporting the common assertion that the GIT microbiota of formula fed infants more closely resembles the adult [328]. While the mechanisms for these differences in microbial colonisation and establishment are not fully understood, it is possible that the numbers of bifidobacteria species in breast fed infants may be enhanced by the oligosaccharides in human milk (which collectively form the third largest solid component of milk (5 to 23g/L) [72] after fat and protein).

Milk oligosaccharides (sugar polymers, typically three to ten units) have been studied because of their marked influence on the GIT microbiota (i.e. prebiotic activity). Human milk contains a variety of oligosaccharides able to stimulate the growth of specific commensal GIT microbiota [132], as well as stimulate the development of the immune system [297], prevent adhesion of pathogens to epithelial tissues [331]. Infant-type bifidobacterial species such as *B. longum* subsp. *longum*, *B. longum* subsp*. infantis*, *B. bifidum* and *B. breve,* for example, contain enzymes specifically involved in the metabolism of human milk oligosaccharides (HMO) [332]. Ruminant milk is common, so ruminant oligosaccharide sources with similar health effects to those in human milk are sought after as possible functional ingredients that could be used in infant formulas, to promote microbiota in the GIT in formula fed infants similar to that of breast fed infants [333].

Modern infant formulas are increasingly supplemented with a mixture of plant derived oligosaccharides (such as FOS and inulin (DP = 10 to 60) [334]), and lactose derived oligosaccharides (1:9) (such as GOS). These elicit an unspecific bifidogenic response [335], and lack the complexity and diversity of HMO, so are unlikely to successfully mimic the structure specific effects of HMO. High concentrations of GOS (16 mg/mL), for example, were required to reduce entheropathogenic *E. coli* adherence to cells in culture [336]. The authors suggested that this anti-adherence activity could be enhanced by fucosylation or sialyation of the GOS. Fucosylated and sialylated HMO are complex oligosaccharides known to act as bacterial adhesin analogues and/or to mimic the receptors used by enteric pathogens to adhere to the surface of host epithelial cells [331], [265].

Sialyloligosaccharides support the growth of breast fed, neonate specific commensal bacteria, such as *Bifidobacterium longum* subsp. *infantis* ATCC15697 [282]. It has been suggested that these acidic oligosaccharides, in addition to enhancing bifidobacterial growth (the “bifidogenic effect”), may combat influenza infections [337] and ulcers caused by *Helicobacter pylori* [284]. Other activities reported include regulation of inflammation by reducing adhesion of human leukocytes on activated endothelial cells [338], and promotion of commensal enteric bacterial

proliferation [237, 285, 286]. Sialyloligosaccharides are found in human milk and are the most prevalent oligosaccharides in caprine colostrum, milk and whey.

Few studies have investigated the biological functions of CMO. CMOP obtained were shown to modulate the expression of goblet cell-specific genes in the HT29/MTX model [268], and to have anti-inflammatory effects in a rat experimental model of colitis [269]. Recent *in vitro* work showed that CMOP were able to increase the concentrations of *Bifidobacterium* spp. from human faeces in anaerobic batch cultures [339].

3.2 Hypothesis and Aims

The hypothesis of the research presented in this chapter was that bifidobacteria isolated from the faeces of breast fed infants are able to ferment CMOP, increasing bifidobacterial growth and SCFA production.

To test this hypothesis, this study aimed to investigate the ability of bifidobacteria strains isolated from exclusively breast fed infants to ferment CMOP (especially sialyloligosaccharides), and to produce SCFA (compared with other prebiotics or milk sugars).

3.3 Material and methods

3.3.1 Isolation of bifidobacteria

Collection of faecal samples from healthy breast fed infants was approved by the local human ethics committee (Massey University, Palmerston North, New Zealand). For the isolation of bifidobacteria, 1 g of fresh faeces was taken from a freshly soiled nappy from four exclusively breast fed infants. Each sample was diluted (10^{-1} to 10^{-7}) with peptone water in a 10 fold dilution series. Peptone water (10 g L^{-1} peptone, 5 g L^{-1} sodium chloride, pH 7.2), is a basal medium, rich in water soluble protein, used as a convenient inoculum for carbohydrate fermentation tests [340]. Each dilution was spread-plated onto modified TPY agar (MTPY) [341], and the plates incubated anaerobically (93% CO_2, 7% H_2) at 37°C for 48 h. Bacterial colonies were then individually picked

and subcultured on fresh MTPY agar plates to obtain single colonies. Five colonies from each faecal sample were subcultured on anaerobic De Man-Rogosa-Sharpe agar (MRS, Oxoid, UK), pH 6.5 - 7.0 supplemented with L-cysteine hydrochloride (0.5 g L^{-1}), at 37°C for 48 h, and kept on anaerobic MRS slopes at -80°C until further analysis.

3.3.2 Bifidobacterial characterisation

The identity of the bifidobacterial isolates was confirmed using 16S rRNA gene sequencing. Each strain was grown in 3 mL MRS liquid culture, as described previously, and DNA extracted from centrifuged cells using a phenol/chloroform/isoamyl alcohol extraction method, followed by precipitation of nucleic acids in isopropanol [342]. From the isolated genomic DNA, the 16S rRNA gene was amplified using bif 164 (5-GGG TGG TAA TGC CGG ATG-3) and bif 662 (5-CCA CCG TTA CAC CGG GAA-3) primers [343]. For PCR, 50 µL reactions were made up containing 1 µL of each primer (stock 10 pM μL^{-1}), 47 µL PCR Supermix (Invitrogen), and 1 µL DNA template. Amplification was undertaken using a PX2 Thermal Cycler (Ashford, UK) with an initial denaturation cycle of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 45 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. Initially 5 µL of each PCR reaction was used to detect the presence of the 498 bp amplicon by agarose gel electrophoresis (Innovative Sciences Ltd, Dunedin, New Zealand). Approximately 0.5 µL 10 X gel loading buffer (Bluejuice, Invitrogen, Auckland New Zealand) was added to the sample before it was run across a 1.0% (w/v) agarose (Boeheringer Mannheim, Germany) gel in Tris acetate/EDTA buffer, pH 8.0. Each gel was run at 180 V (approximately 15 V/cm for 55 min, and stained using ethidium bromide (0.5 µg mL^{-1}). The remaining PCR products were purified to remove residual primers and dNTPs using the QIAquick PCR Purification Kit (Qiagen, Auckland, New Zealand), and labelled for sequencing using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Auckland, New Zealand). The Massey Genome Service (Massey University, Palmerston North, New Zealand) sequenced the PCR product (15 ng) on a ABI3730 (Applied Biosystems, Auckland, New Zealand) using the bif 164 or bif 662 primers (3.2 pM). Traces

were aligned using Contig Express (Vector NTI Advance, Version 11.0, Invitrogen Corporation, California, USA) and the 16S rRNA gene sequences compared with known bacterial sequences available from GenBank database using BLAST.

3.3.3 Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD, [344]) is the most frequently used tool for discrimination between microbial species and strains [345], and for assessing the genetic variability of the investigated isolates. The RAPD method has been used for detection and identification of some Bifidobacterium strains isolated from human gastrointestinal tract and food samples [346, 347].

RAPD PCR was performed using seven random decamer primers: P2 5´-GAT CGG ACG G-3´, P15 (5′ CTG GGC ACG A 3′), P16 (5′ TCG CCA GCC A 3′), P17 (5′CAG ACA AGC C 3′) [348], PER1 (5′AAG AGC CCG T 3′) [349], and CC1 (5′AGC AGC GTG G 3′) [350], CORR1 5´-TGC TCT GCC C-3´ [351]. The amplifications were carried out in a total volume of 25 µL containing 100 ng template DNA, 1× PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 1 µL of each primer (stock 10 pM μL^{-1}), 1.5 units of Platinum Taq DNA polymerase (Invitrogen), and sterile molecular biology grade water to 25 µL. Amplification reactions were carried out using a PX2 Thermal Cycler (Ashford, UK) with the following cycling parameters: 4 min, 94°C; 35 cycles 1 min, 94°C; 1 min, 36°C; 2 min, 72°C; last elongation step 7 min, 72°C. The RAPD PCR products (3 µL) were separated by electrophoresis on a 1.5% (w/v) agarose gel and DNA stained using ethidium bromide (0.5 µg mL^{-1}). A dendrogram was generated from RAPD profiling with 7 random primers using Bionumerics 4.0 (Applied Maths, TX, USA). Profiles were compared using Dice's Similarity Coefficient at a tolerance of 2%.

3.3.4 Amplified ribosomal DNA restriction analysis (ARDRA) of *Bifidobacterium longum* subsp *longum* versus *Bifidobacterium longum* subsp *longum infantis*.

PCR oligonucleotide primers Pbi F1 (5'-CCG GAA TAG CTC C-3') and Pbi R2(5'-GAC CAT GCA CCT GTG AA-3') were used to amplify a 914 bp DNA fragment to differentiate *B. longum* subsp. *longum* from *B. longum* subsp. *infantis* by restriction endonuclase digestion with Sau3AI (New England Biolabs, Massachusetts, USA) [352]. PCR amplicons were digested with two units of Sau3AI for 2 h at 37°C. ARDRA digests (5 µL) were then separated by agarose (2% (w/v)) gel electrophoresis. Gels were stained in ethidium bromide (0.5 µg mL^{-1}) and ARDRA DNA profiles visualised on a transilluminator (Kodak; Biolab, Auckland, New Zealand).

3.3.5 *In vitro* carbohydrate fermentation and short chain fatty acid analysis of isolated bifidobacterial strains

To investigate the growth profiles of bifidobacterial strains, each strain was inoculated into MRS broth supplemented with 0.05% (w/v) L-cysteine and incubated at 37°C for 36 h until late logarithmic/early stationary phase was reached (determined previously from growth curves). 100 µL was then subcultured into a defined semi-synthetic medium [353] lacking a carbohydrate source (negative control) or supplemented (final concentration 1% [w/v]) with CMOP (5 g/L CMO, 1 g/L GOS, 2 g/L lactose, 1 g/L glucose and 1 g/L galactose), combo (1 g/L GOS, 2 g/L lactose, 1 g/L glucose and 1 g/L galactose), or 10 g/L of glucose (BDH), lactose (BDH), oligofructose P95 (BENEO-Orafti, Invita NZ Ltd., Auckland, New Zealand; average DP 4), inulin HP (BENEO-Orafti, Invita NZ Ltd., Auckland, New Zealand; average DP 25), GOS (TOS-100, Yakult Japan; DP < 8), 3'-syalyl-lactose (Carbosynth, UK), or 6'-syalyl-lactose (Carbosynth, UK) as the sole carbohydrate source. The dominant oligosaccharides present in the CMOP were 3'- and/or 6'-galactosyl-lactose (12%, DP = 3), 3'- and/or 6'-sialyl-lactose (27%, DP= 3), 6'-glycolyl-neuraminyl-lactose (32%, DP= 3), lacto-*N*-hexaose (9%, DP= 6), disialyl-*N*-lactose (11%, DP= 4), 6'-*N*-acetyl-glucosaminyl-lactose (7%, DP = 3). Each bifidobacterial strain was grown in triplicate in 5 mL of semi-synthetic

broth, and cultured anaerobically for 45 h at 37°C. Cell growth was measured 2 hourly using optical density (OD) at 600 nm on a spectrophotometer (Ultrospec 1100 pro, Amersham Biosciences).

Fermentation reactions from six strains (two *B. bifidum*, two *B. longum*, and two *B. breve*), selected for their optimum growth in CMOP supplemented medium, were evaluated for SCFA production. Anaerobic growth of each strain in six different broth cultures where glucose, CMOP, combo, lactose, 3'-sialyl-lactose or 6'-sialyl-lactose was the sole carbohydrate source, was assessed in triplicate at 37°C. At 16 h (mid logarithmic phase) and 36 h (stationary phase) after inoculation, broths were centrifuged for 10 min at 3000 x g at 4°C, and the pH measured using a PHM62 pH meter (Radiometer Pacific). Aliquots of the fermentation liquid were filtered using 0.22 µm filters (Millipore Australia Pty Ltd, NSW, Australia) and the concentration of formic acid, acetic acid, propionic acid, isobutyric acid (2-methylpropanoic acid), butyric acid, isovaleric acid (3-methylbutanoic acid), valeric acid (pentanoic acid), and caproic acid (hexanoic acid) were determined using a Shimadzu RID 10A HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand) fitted with a Bio-Rad Aminex 87H 9 µm, 7.5 x 300 mm HPLC column (maintained at 45˚C). The mobile phase was sulphuric acid (5 mM) using an isocratic elution with a flow rate of 0.8 mL min^{-1}. The injected volume was 50 µL and the run time was 35 min between injections. SCFA concentrations in the samples were quantified using standards (Shimadzu Oceania, Pickering Laboratories, USA), and LC Solution ver. 1.22 SP1 software (Shimadzu, Kyoto, Japan).

3.3.6 Utilisation of caprine milk oligosaccharide enriched fraction by bifidobacteria

The utilisation of CMO, GOS, lactose, glucose and galactose present in the CMOP was assessed in six bifidobacterial strains (two *B. bifidum*, two *B. longum*, and two *B. breve* strains) selected on the basis of contrasting growth profiles. Each strain was incubated in triplicate, and culture supernatants (50 µL of each tube) taken immediately after inoculation, and at stationary phase (36 h), were analysed by LC-MS and HPLC to evaluate the depletion of the different carbohydrates. The depletion of specific oligosaccharides present in CMOP was analysed by the intensity of their

specific masses using LC-MS data and reported as percentage of depletion compared to the concentrations in the control. The HPLC and LC-MS methods are described in Chapter 2.

3.3.7 Bifidobacterial exo-α-sialidase genes

The presence of sialidase genes described for *B. longum* and *B. bifidum* [354, 355] and a putative *B. breve* (NC_017218.1) sialidase gene were evaluated in the 17 bifidobacterial strains. PCR primers were designed to amplify the DNA sequence encoding the glycosyl hydrolase family 33 domain of the sialidase encoding genes (Table 3.1A and B) from *B. longum*, *B. bifidum* and *B. breve*. PCR products were amplified, viewed, purified and sequenced using the PCR primers as sequencing primers as described previously. The *B. longum* subsp. *infantis* strain JCM 10088, control DNA was kindly gifted by Professor Gerald Tannock (University of Otago, New Zealand).

3.3.8 *In vitro* sialidase assay

The method of Kiyohara *et al* [356] (incorporating the fluorescent substrate 4-methylumbelliferyl-a-D-*N*-acetylneuraminic acid (4MU-Neu5Ac) (Sigma, Auckland, New Zealand)), was adapted to determine specific sialidase activity of selected bifidobacteria. Briefly, bifidobacterial strains were grown anaerobically in 5 mL of a semi-synthetic medium [353] supplemented with CMOP, combo, or 10 g L^{-1} of 3'-syalyl-lactose (Carbosynth, UK) or 6'-syalyl-lactose (Carbosynth, UK) as the sole carbohydrate source for 24 h at 37°C. Six separate cultures of each of two *B. bifidum*, two *B. longum*, and two *B. breve* strains were examined, with each biological replicate assessed in triplicate. Stationary phase bacterial cells were collected by centrifugation (3000 x g, 10 min, 4°C), washed three times in 50 mM sodium phosphate buffer (pH 7.0), and adjusted to an OD_{600} of 0.1. Culture fluids from each treatment were collected and kept at 4°C until analysed. 4MU-Neu5Ac was prepared in the same buffer at a concentration of 100 µg/mL and stored at -20°C in small aliquots. Assays were prepared by thoroughly mixing 20 µL of substrate solution with 50 µL of bacterial suspension or culture fluid. After a 30 min incubation at 37°C, the reaction was stopped by adding an equal volume of 0.5 mole/L sodium carbonate (pH 11.6) to the reaction and the

Table 3.1 Sialidase genes and primers used to screen for sialidase domains in the bifidobacterial isolates. (A) Sialidase protein characteristics, (B) predicted cellular localisation, based on the presence of export signal, transmembrane domain or cell wall anchor motifs and primers .

A)

Origin	GenBank accession number	Protein name	Length (aa)	GH33 region (aa)	Signal peptide cleavage site[a]	Transmembrane helices[b]
B. longum subsp. *infantis* ATCC15697	ACJ53406	Blon_2348	394	35-368	None	None
B. breve ACS-071-V-Sch8b	AEF27628	HMPREF9228_0182	763	325-730	None	None
B. bifidum PRL2010	ADP36806	BBPR_1793	1795	325-650	Between aa 39-40	2 between aa17-39 and aa1767-1789
B. bifidum PRL2010	ADP36807	BBPR_1794	834	190-510	Between aa 35-36	2 between aa13-35 and aa806-828

[a] [357]

[b] TMHMM Server v. 2.0, http://www.cbs.dtu.dk/services/TMHMM/

B)

Origin	Predicted localisation	Primer name	Sequence	Amplicon length	Region amplified
B. longum subsp. *infantis* ATCC15697	Intracellular	Sialil-Inf	F. 5'-TACTGTGTGCGGCGCGAACC R. 5'-CAGACAGCGGAAAACCGCCGA	1136bp	33-1168bp
B. breve ACS-071-V-Sch8b	Intracellular	Sialil-Br	F. 5'-GCGGTGCGGTGGACATCTAT R. 5'-CAGCCGACTTCACTCCGAA	1527bp	737-2263bp
B. bifidum PRL2010	Extracellular	Sialil-Bif5	F. 5'-GCGACCACTCAGGACGGCAC R. 5'-TCCGAGATCGCAACGCGACG	1198bp	448-1645bp
B. bifidum PRL2010	Extracellular	Sialil-Bif2	F. 5'-GCTGCATGCGGTCGTCGTCA R. 5'-TCGTGGCGTTGGCATTCGCA	1049bp	915-1963bp

fluorescence from 4-methylumbelliferyl was measured (FlexStation 3 Benchtop Microplate Reader, Bio-Strategy) using activation and excitation wavelengths of 360 and 460 nm, respectively. Enzyme activities were determined by comparing absolute fluorescence units (afu) minus the blank, as described by O'Brien & Mitsuoka [358].

3.3.9 Statistical analysis

Bacterial growth, SCFA profiles and sialidase production at 36 h of fermentation for each substrate were tested for normality and homogeneity of variances and compared by one-way analysis of variance (ANOVA) using GenStat (15th edition SPS). Differences were considered significant at $P \leq 0.05$. To identify the correlation between bacterial growth, pH and SCFA production, Pearson's Rank correlation factors and P values were calculated using GenStat (15th edition SPS). Correlations were considered significant if $P \leq 0.05$.

3.4 Results

3.4.1 Genetic characterisation of bifidobacterial strains

A total of 17 bifidobacterial strains were isolated from faecal samples of four exclusively breast fed infants. The strains were positively identified by amplification and sequencing of a 498 bp region of the 16S rRNA gene corresponding to the V2 to V3 variable regions as *B. bifidum* (n = 4), *B. longum* (n = 6) and *B. breve* (n = 7) (Figure 3.1). For the six *B. longum* strains, the 16S rRNA gene was amplified using the genus specific primer set (Pbi F1/Pbi R2), and digested with the enzyme Sau3AI. ARDRA of genus specific amplicons revealed a DNA fragment pattern consistent with *B. longum* subsp. *longum* for all the strains tested.

RAPD analysis of the 17 bifidobacterial strains was undertaken to further characterise the isolated strains to explain any phenotypic variations. The dendrogram separated the 17 strains into four main clusters, broadly corresponding to the three different Bifidobacteria species (Figure 3.1). A clear distinction between *B. longum ssp longum* isolated from infant 2 (AGR2170 to

Cluster/group	Species	Strain number	Infant	Sialidase gene PCR amplification			
				Blon_2348 *B. infantis* ATCC 15697 (NC_011593.1)	BBPR_1794 *B. bifidum* PRL2010 (NC_014638.1)	BBPR_1793 *B. bifidum* PRL2010 (NC_014638.1)	BNR/Asp-box *B. breve* ACS-071-V-Sch8b (NC_017218.1)
A	*B. bifidum*	AGR2166*	1	-	+	+	-
		AGR2165	1	-	+	+	-
		AGR2167	1	-	+	+	-
		AGR2168*	1	-	+	+	-
B	*B. longum* subsp. *longum*	AGR2173*	2	-	-	-	-
		AGR2170	2	-	-	-	-
		AGR2171	2	-	-	-	-
		AGR2172	2	-	-	-	-
		AGR2174	2	-	-	-	-
C		AGR2176*	3	-	-	-	-
D	*B. breve*	AGR2179	4	-	-	-	+
		AGR2177*	3	-	-	-	+
		AGR2181	4	-	-	-	+
		AGR2178	3	-	-	-	+
		AGR2169	1	-	-	-	+
		AGR2183	4	-	-	-	+
		AGR2175*	3	-	-	-	+

Figure 3.1 Seventeen bifidobacterial strains isolated from four exclusively breast-fed infants were identified based on their 16S rRNA gene sequences. Dendrogram showing the relationships between the analysed bacterial strains based on Dice similarity coefficients calculated from random amplification polymorphic DNA analysis data. The bar at the top of the diagram indicates the similarity index (maximum of 100%). PCR amplification and sequence analysis of sialidase genes was tested in all seventeen bifidobacterial strains. (+) Identity with control sequence; (-) no amplification, or non-specific amplicon generated. * Strains chosen for further characterisation by short chain fatty acid production and carbohydrate metabolism.

AGR2174) and 3 (AGR2176) was discernible by RAPD analysis and some inter-strain heterogeneity among isolates was observed.

3.4.2 *In vitro* fermentation profile and short chain fatty acids analysis

All 17 strains were assessed for growth in media supplemented with glucose, CMOP (50% oligosaccharides, 10% GOS, 20% lactose, 10% glucose and 10% galactose), combo, oligofructose, lactose, GOS, 3'-sialyl-lactose, 6'-sialyl-lactose, or no carbohydrate (Figure 3.2). No significant growth (OD_{600} < 0.5) was observed from any bifidobacterial strains in the medium supplemented with inulin or with no added carbohydrate (data not shown).

In general, all strains reached their highest optical densities at 16 h with CMOP as the sole carbohydrate source, compared to all the other carbohydrate sources (P < 0.001). The oligosaccharides 3'-sialyl-lactose and 6'-sialyl-lactose were fermented only by *B. bifidum* strain AGR2166 (Figure 3.2 a). *B. bifidum* strain AGR2166 fermented all tested carbohydrate substrates except oligofructose. The remaining *B. bifidum* strains (AGR2165, AGR2167 and AGR2168), were only able to ferment CMOP as a carbohydrate source (Figure 3.2 b). The *B. longum* subsp *longum* AGR2170, AGR2171, AGR2172, AGR2173, AGR2174 (Figure 3.2 c), AGR2176 (Figure 3.2 d) and *B. breve* AGR2169, AGR2177, AGR2178, AGR2179, AGR2181, AGR2183 (Figure 3.2 e), AGR2175 (Figure 3.2 f) strains reached an intermediate optical density (OD_{600} = 1.5) with oligofructose, and increased optical densities (OD_{600} ≥ 2) with GOS, lactose, combo and glucose as the sole carbohydrate source, but with a different growth profile during the 48 h of fermentation.

A single strain representative of each fermentation profile was selected for quantifying SCFA production (Figure 3.2, *B. bifidum* AGR2166 (a), AGR2168 (b); *B. longum* AGR2173 (c), AGR2176 (d); *B. breve* AGR2177 (e), AGR2175 (f)). Only acetic and lactic acid were produced after 16 h and 36 h incubation for all the strains tested (Figure 3.3). All strains (except AGR2168), produced higher concentrations (P < 0.001) of acetic and lactic acid in a medium supplemented with CMOP at 36 h of growth compared to the medium supplemented with combo (Figure 3.3) and other

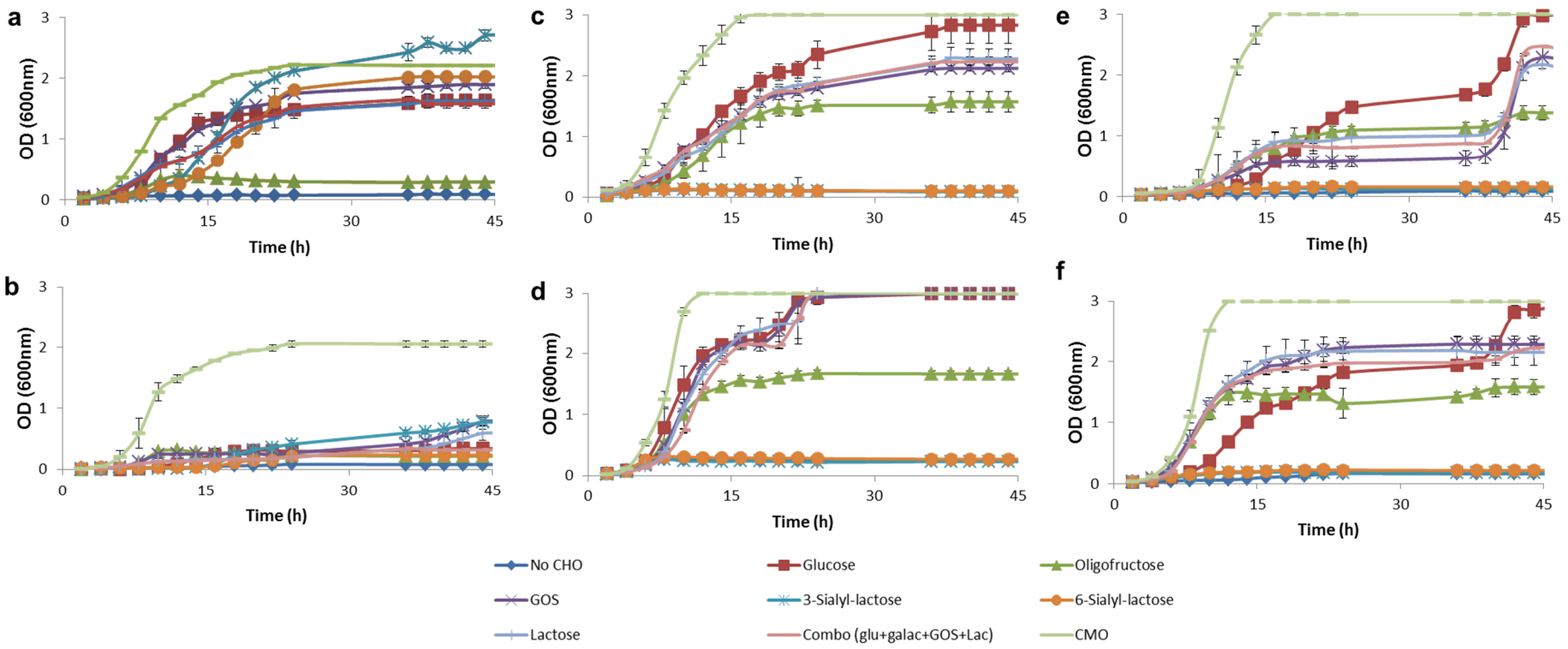


Figure 3.2 Growth profile of (a) *B. bifidum* AGR2166; (b) *B. bifidum* AGR2168 (similar to AGR2165 and AGR2167); (c) *B. longum* AGR2173 (similar to AGR2170, AGR2171, AGR2172 and AGR2174); (d) *B. longum* AGR2176; (e) *B. breve* AGR2177 (similar to AGR2169, AGR2178, AGR2179, AGR2181, AGR2183); (f) *B. breve* AGR2175.. Each time point is an average of three replicates and the errors bars indicate standard deviation.

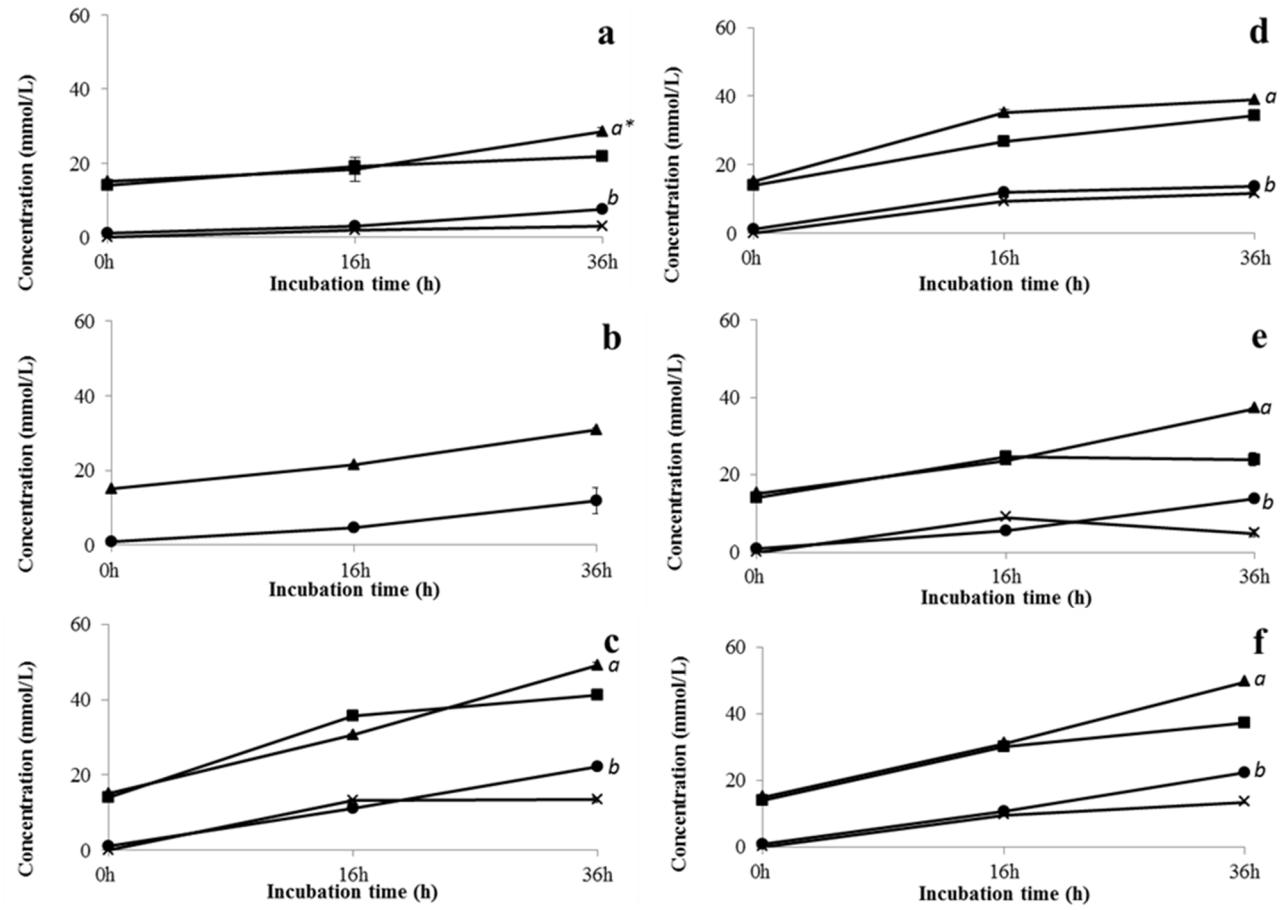


Figure 3.3 Acetic and lactic acid produced by bifidobacterial strains; (a), *B. bifidum* AGR2166; (b), AGR2168; (c), *B. longum* AGR2173; (d), AGR2176; (e), *B. breve* AGR2177; and (f), AGR2175. Each strain was grown in triplicate in semi-synthetic broth supplemented with 1% caprine milk oligosaccharides (determined from caprine milk oligosaccharide enriched fraction). Short chain fatty acids were measured at 0 h, 16 h and 36 h of growth and compared to media supplemented with combo. ▲, acetate produced by caprine milk oligosaccharides enriched product fermentation; ■, acetate produced by combo fermentation; ●, lactate produced by caprine milk oligosaccharides enriched product fermentation; ×, lactate produced by combo fermentation. Each short chain fatty acids data point is an average of three replicates and the errors bars indicate standard deviation. * [a] Higher concentrations of acetate compared to combo ($P < 0.001$). [b] Higher concentrations of lactate compared to combo ($P < 0.001$).

carbohydrates. AGR2168 only grew in the medium supplemented with CMOP, thus comparisons were not possible. Production of acetic and lactic acid by all the strains tested was associated with a decrease in overall pH of the culture over 36 h with the CMOP (Figure 3.4). At 36 h, AGR2166 and AGR2168 had higher pH value compared to AGR2173, AGR2175, AGR2176 and AGR2177 (AGR2166, 4.89 ± 0.005; AGR2168, 4.74 ± 0.13; AGR2173, 4.23 ± 0.05; AGR2175, 4.21 ± 0.05; AGR2176, 4.53 ± 0.02; AGR2177, 4.29 ± 0.05; mean ± SD, $p<0.001$). The drop in pH from 6.5 to 4.2 - 4.9 was correlated with the final OD of the culture ($r= -0.95$; $P = 0.001$), and the concentration of acetic ($r= -0.85$; $P=0.02$) and lactic acid ($r= -0.83$; $P = 0.03$) produced. No significant difference in SCFA production was found at 16h.

3.4.3 *In vitro* bifidobacterial catabolism of oligosaccharides from caprine milk oligosaccharides enriched fraction

The initial relative abundance of several oligosaccharides associated with the CMOP, measured by LC-MS, was approximately 13% 3'- and/or 6'-galactosyl-lactose, 27% 3'- and/or 6'-sialyl-lactose, 32% 6'-Glycolyl-neuraminyl-lactose, 9% lacto-*N*-hexaose and less than 11% disialyl-*N*-lactose and 8% 6'-*N*-acetyl-glucosaminyl-lactose. All strains preferentially catabolised the 3'- and/or 6'-galactosyl-lactose and 3'- and/or 6'-sialyl-lactose (Figure 3.5). *B. bifidum* AGR2166 had the highest levels of depletion (35 to 55%) of 3'- and/or 6'-galactosyl-lactose, 3'- and/or 6'-sialyl-lactose and 6'-glycolyl-neuraminyl-lactose. Disialyl-*N*-lactose and 6'-*N*-acetyl-glucosaminyl-lactose had the lowest depletion levels (<10%, similar among all strains). The depletion rate of all oligosaccharides, however, was different between strains from the same species (Figure 3.5). *B. bifidum* AGR2166, for example, had the higher catabolism level among all strains, removing 33% of the total oligosaccharides identified in the CMOP, more pronounced then the reduction by *B. bifidum* AGR2168 (13%). A similar strain dependant variation of oligosaccharide catabolism was noted for *B. longum* subsp *longum* AGR2173 (7%), AGR2176 (14%) and *B. breve* AGR2175 (5%) and AGR2177 (16%) strains.

The concentrations of galactose, glucose, lactose, and GOS in a media supplemented with 1%

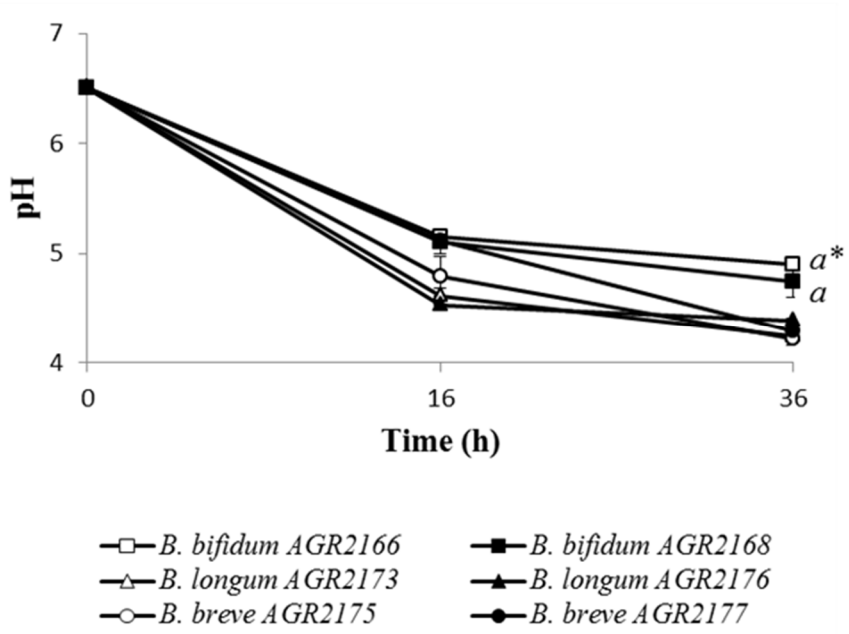


Figure 3.4 Change in pH associated with fermentation of caprine milk oligosaccharides enriched product by *B. bifidum* (AGR2166 and 2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains. Each pH measurement is an average of three replicates and the errors bars indicate standard deviation. * [a] pH significantly higher ($P < 0.001$) at 36h compared to the other strains.

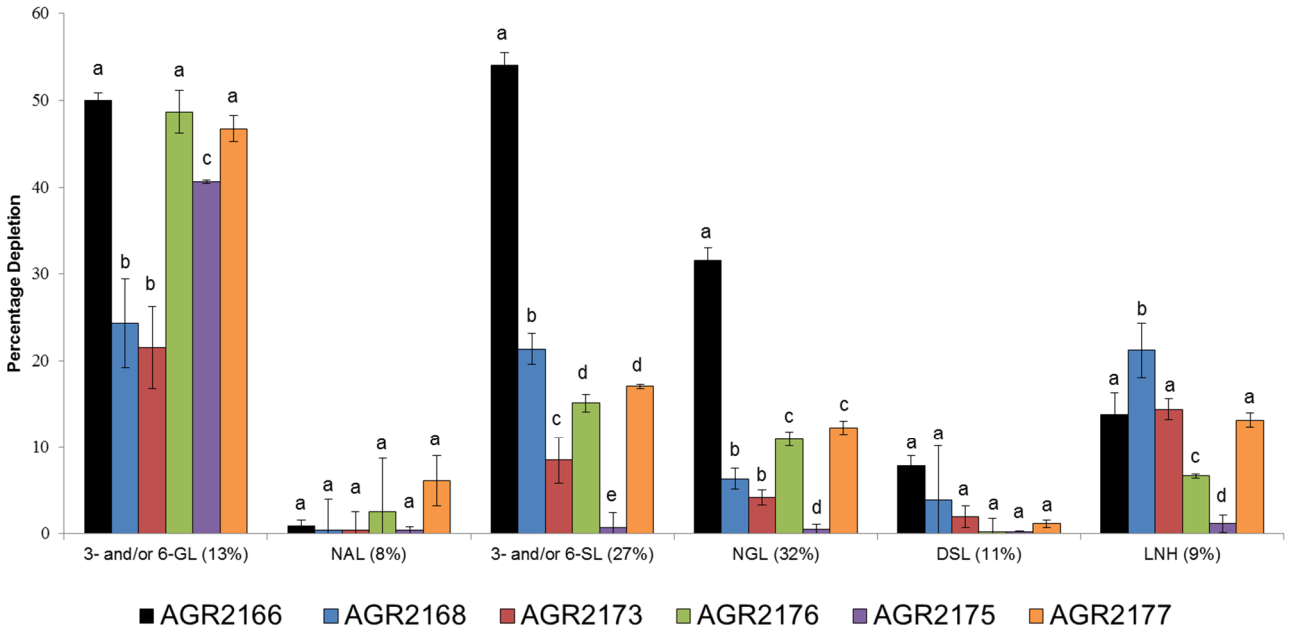


Figure 3.5 Percentage of oligosaccharide depletion by *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains after 36 h of growth in semi-synthetic broth supplemented with 1% of caprine milk oligosaccharides, when compared to the uninoculated control media. The oligosaccharides are represented by their abbreviation and their relative initial abundance is shown in brackets. 3'- and/or 6'-galactosyl-lactose (3-GL and/or 6-GL), 3'- and/or 6'-sialyl-lactose (3-SL and/or 6-SL), 6'-glycolyl-neuraminyl-lactose (NGL), lacto-*N*-hexaose (LNH), disialyl-*N*-lactose (DSL), 6'-*N*-acetyl-glucosaminyl-lactose (NAL). Each strain was incubated in triplicate and errors bars show the standard deviation of depletion. Comparing each oligosaccharide, bars with dissimilar letters differ significantly in depletion ($P<0.001$)

CMOP were determined after 36 h of bifidobacterial incubation and compared to the concentrations immediately after inoculation (Figure 3.6). These data indicate that lactose was depleted by all bifidobacterial strains tested. The degradation of lactose, GOS, and CMOP were likely to have increased the overall concentrations of glucose and galactose in the media, which were not fully utilised by the end of the 36 h incubation. *B. bifidum* AGR2166 and AGR2168 had lower levels of glucose and higher levels of galactose in their media compared to pre-incubated media and to the other strains after CMOP fermentation. AGR2166 also had higher levels of lactose, compared to other strains, which may have been associated with oligosaccharide catabolism by this strain. The GOS concentration in the *B. longum* AGR2173 and *B. breve* AGR2175 media after incubation did not differ from uninoculated media; however, 50% of the GOS present in the pre-incubated media was fermented by the other strains.

3.4.4 Identification of genes encoding for exo-α-sialidase and associated sialidase activity

Sialidase activity of the bifidobacterial isolates on CMO was assessed using molecular methods to demonstrate the presence of sialidase-encoding genes and by measuring sialidase activity using a fluorogenic substrate. The presence of three reported sialidase genes (BBPR_1793 and BBPR_1794 from *B. bifidum* PRL2010 and HMPREF92 28_0182 from *B. breve* ACS-071-V-Sch8b), were confirmed by PCR amplification and DNA sequencing in the *B. bifidum* and *B. breve* strains from this study (Figure 3.1). Sialidase enzyme activity was also analysed in six selected isolates (highlighted in Figure 3.1); two *B. breve*, two *B. bifidum* and two *B. longum*. Only the *B. bifidum* isolates (AGR2166 and AGR2168) had cellular sialidase activity when grown in the presence of CMOP (Figure 3.7), although *B. bifidum* AGR2168 had only limited ability to ferment the CMO sialyloligosaccharides, 3'- and 6'-sialyl-lactose as a sole source of carbon (Figure 3.2). Sialidase activity from *B. bifidum* was mainly cell-associated, although approximately 12 % residual activity was also detected in the culture supernatant of these two strains (Figure 3.7 A). *B. bifidum* cellular sialidase activity in AGR2166 and AGR2168 was induced by all four substrates examined,

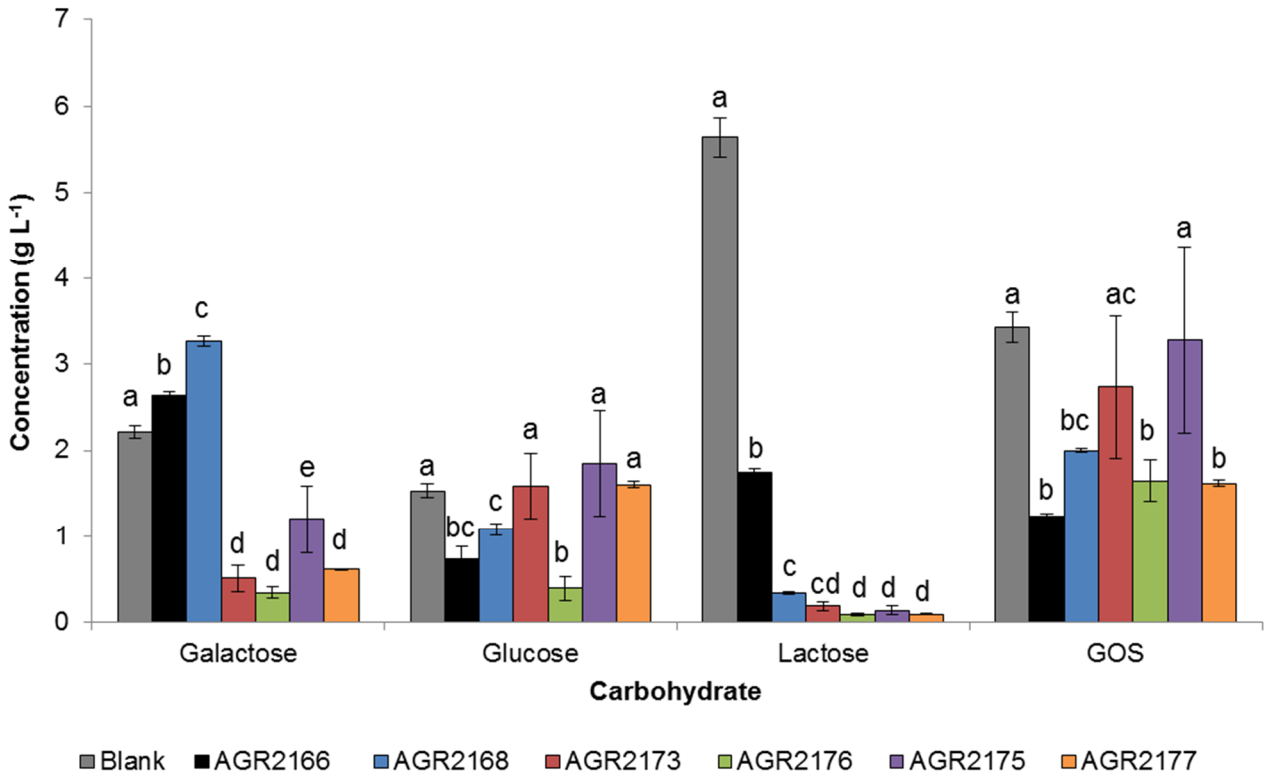


Figure 3.6 Total residual carbohydrate remaining in semi-synthetic media supplemented with 1% of caprine milk oligosaccharides after 36 h of fermentation by *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains. Each strain was incubated in triplicate and errors bars show the standard deviation of remaining carbohydrate. Bars with dissimilar letters differ significantly (P<0.001), within each carbohydrate.

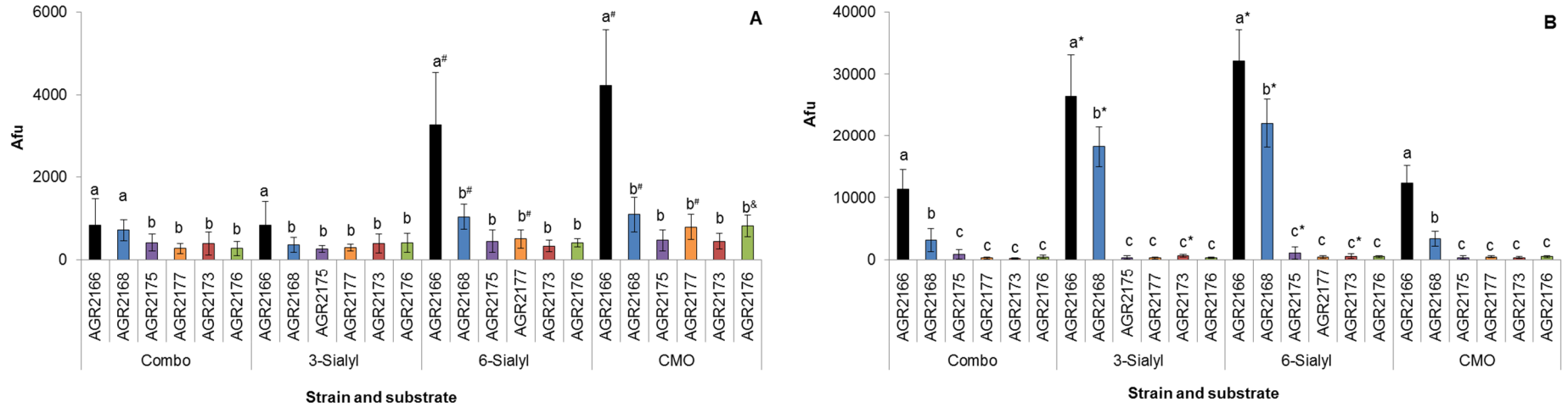


Figure 3.7 Sialidase activity associated with *B. bifidum* (AGR2166, black; and AGR2168, blue), *B. longum* (AGR2173, purple; and AGR2176, orange) and *B. breve* (AGR2175, red; and AGR2177, green) strains after growth in semi-synthetic media supplemented with 1% (w/v) final concentration of combo, 3'-sialyl-lactose, 6'-sialyl-lactose and caprine milk oligosaccharides. Sialidase activity associated with culture supernatant and cells alone was measured by absolute fluorescence units (afu) produced by 4-methylumbelliferone released during MUN hydrolyses. (A) Culture fluid sialidase activity; (B) Cellular sialidase activity. Six separate cultures of each of two *B. bifidum*, two *B. longum*, and two *B. breve* strains were examined, with each biological replicate assessed in triplicate. Bars with dissimilar letters differ significantly ($P < 0.001$), within each substrate. *Substrate with increased sialidase activity ($P \leq 0.01$) compared to caprine milk oligosaccharides enriched product and combo. # Caprine milk oligosaccharides enriched product and 6'-sialyl-lactose with increased sialidase activity compared to combo and 3'-sialyl-lactose ($P \leq 0.001$). $^{\&}$ Caprine milk oligosaccharides enriched product with increased sialidase activity compared to the other substrates ($P < 0.001$).

and was significantly enhanced ($P \leq 0.01$) in bacterial cell preparations taken from cultures grown in the presence of 3'- and 6'-sialyl-lactose when compared to CMOP and combo. Despite the presence of 3' and 6'-sialyl-lactose in CMOP, there was no increase in sialidase activity of AGR2166 and AGR2168 grown in the presence of CMOP compared to the combo (Figure 3.7B). Similar sialidase activity was observed from the culture supernatant taken from AGR2166 grown in the presence of 6'-sialyl-lactose and CMOP, and this sialidase activity was higher than combo and 3'-sialyl-lactose ($P < 0.001$). Despite the presence of a sialidase-encoding gene in the *B. breve* isolates examined, no sialidase activity was observed. However, in contrast to the sialidase proteins from *B. bifidum* that have signal peptide cleavage sites and transmembrane helices, no such structural characteristics were associated with the sialidase from *B. breve,* suggesting a potential intracellular localisation. Intracellular sialidase activity was not determined.

3.5 Discussion

This study investigated the *in vitro* effects of a CMOP on the growth of selected bifidobacteria isolated from four exclusively breast fed infants. The CMOP (containing high concentrations (46%) of sialyloligosaccharides), supported enhanced growth of selected bifidobacteria strains isolated from breast fed infants, and stimulated the *in vitro* production of SCFA, such as acetate and lactate. These results confirm the hypothesis, *Bifidobacteria isolated from the faeces of breast fed infants are able to ferment CMO, increasing bifidobacteria growth and metabolism.*

In a recent study, CMO was shown to increase the growth of human faecal *Bifidobacterium* spp in anaerobic batch culture [339], although the specificity of bifidobacterial CMO consumption was not investigated. The bifidobacterial strains isolated in this work, *B. breve*, *B. longum* and *B. bifidum*, together with *B. longum* subsp *infantis* and *B. adolescentis*, are among the most prevalent species found in infants independent of their feeding regime [93, 330, 359, 360]. Although only a small number of bifidobacterial strains were selected from each infant, previous work suggests that the infant microbiota in the GIT is highly heterogeneous and dominated by 3-5 different bifidobacterial strains [330, 335]. The RAPD analysis undertaken in this study broadly agrees with the well-

recognised level of clonal heterogeneity demonstrated among the *B. breve*, *B. longum* and *B. bifidum* strains as determined by genetic fingerprinting methods such as ribosomal intergenic spacer analysis (RISA) and RAPD [330, 360, 361].

Among the bifidobacterial species tested, *B. bifidum* (AGR2166) was shown to utilise both 3'- and 6'-sialyl-lactose as a sole carbon source to support growth (Figure 3.2) with associated depletion of these same oligosaccharide isomers present in CMOF (Figure 3.5). Enhanced depletion of 3'- and 6'-sialyl-lactose from CMOF by *B. bifidum* (AGR2166) was likely through cell-associated sialidase expression after induction with the same oligosaccharides (Figure 3.7). These data largely agree with previous work [356] that suggests a surface or intracellular location for the sialidase enzyme on *B. bifidum* and that the residual activity associated with the culture supernatant found in this study may be associated with cell wall debris and/or released enzyme present within the culture supernatant. In contrast, *B. bifidum* (AGR2168) displayed intermediate growth on 3'- sialyl-lactose (Figure 3.2b) with partial (20%) utilisation of 3'- and 6'-sialyl-lactose from CMOF (Figure 3.5) with cell-associated sialidase expression (Figure 3.7).

B. longum and *B. breve* strains were unable to utilise 3'- and 6'-sialyl-lactose as a growth substrate when included as the only carbon source (Figure 3.2c to f) and no expression of cell-associated sialidase was observed (Figure 3.7). Limited depletion (5- 15%) of these oligosaccharides by *B. longum* and *B. breve* strains was detected (Figure 3.5) however, when grown in a CMOF enriched media. This partial depletion may have occurred through incomplete catalysis of 3'- and 6'-sialyl-lactose without fermentation of any resulting breakdown products or that these sugars were selectively adsorbed to the bacterial cells that were present in the media, which were then removed prior to analysis. [362] Contrasting oligosaccharide depletion observed between strains of the same species was in accordance with inter-strain heterogeneity shown in the RAPD analyses. *B. breve* AGR2175 and AGR2177, for example, both isolated from the same infant (3) and with similar growth profiles (Figure 3.2), showed different oligosaccharides catabolism profiles (Figure

3.5) which might indicate differential regulation or expression of enzymes involved in carbohydrate metabolism.

Augmented microbial biomass associated with enhanced growth and fermentation of CMOP increased microbial fermentation end products such as acetate and lactate. These data agree with a previous study, where CMO was shown to increase the production of acetate, lactate and propionate in anaerobic batch culture inoculated with human faeces [339]. *In vivo*, SCFA are rapidly absorbed by the colonic mucosa and contribute towards the energy requirements of the host [363]. They also acidify the luminal pH, which suppresses the growth of pathogens [364, 365], and influence intestinal motility [366]. Acetate is mainly metabolised in human muscle, kidney, heart, brain and liver. It plays a role in lipogenesis and contribute to inhibition of cholesterol synthesis [367]. Lactate does not accumulate in the human GIT and may be transformed to acetate, butyrate and/or propionate by the GIT microbiota [368].

An absolute measurement of CMO utilisation in this study was impossible due to the high concentrations (50%) of lactose, GOS, glucose and galactose in the CMOP. However, when used as a sole carbohydrate source, lactose, GOS, glucose and galactose did not support enhanced bacterial growth when compared to the CMOP. It is likely that CMO is not only a fermentable substrate, but also stimulates the utilisation of other simpler carbohydrates. The GlcNAc-containing oligosaccharides (6'-*N*-acetyl-glucosaminyl-lactose and lacto-*N*-hexaose), for example, have been reported as a growth factor stimulating lactose utilisation by *B. bifidum* [369]. The mechanism through which these oligosaccharides are used remains to be identified, but studies on the utilisation of HMO may provide some clues [132, 370]. Certain bifidobacterial strains such as *B. bifidum* NCIMB41171 have the ability to synthesise long chain carbohydrates (such as GOS) from lactose and galactose using the transglycosylic activity of β-galactosidase [371]. Thus, it is difficult to precisely determine how much lactose was degraded to glucose and galactose through the hydrolytic activity of β-galactosidase, and how much GOS, if any, was produced by transglycosylic activity of β-galactosidase [372, 373].

Lactose, the core of all HMO and CMO, and the main structure of galactosyl-lactose, is likely to be degraded to galactose and glucose in a catabolic reaction that requires β-galactosidase activity. *B. bifidum*, for example, contains both extracellular and intracellular β-galactosidases [374]. *B. breve* [372] and *B. longum* subsp. *longum* [374], on the other hand, have been reported to contain only intracellular β-galactosidases, and the high utilisation of lactose by these strains may indicate that lactose is likely to be actively transported into the cells by a yet unidentified transporter. Although GOS is also hydrolysed to glucose and galactose by β-galactosidase, different strains have been shown to have differential consumption of selected GOS with different DPs [375]. The infant isolates (*B. longum* subsp. *infantis* and *B. breve*) are able to more efficiently consume the GOS species with DP from 3 to 8, while *B. adolescentis* and *B. longum* subsp. *longum* exhibited differential consumption of selected DP [375]. These contrasting HMO and CMO utilisations suggest that niche specific adaptation abilities exist among various bifidobacterial species and strains, and with other components of the microbiota of the GIT. These complex effects cannot be reproduced by simple carbohydrate structures most often used as prebiotics.

The ability of the bifidobacterial strains to utilise the different carbohydrates present in CMOP and/or stimulate the consumption of other carbohydrate sources is important to determine the effects of these milk components in the GIT microbiota. Although more than 8% of the identified genes from bifidobacterial genomes are predicted to be involved in carbohydrate metabolism, the ability to metabolise certain complex milk oligosaccharides is species and strain specific [354, 376]. Analysis of the genes involved in carbohydrate utilisation indicate that *B. bifidum* (JCM1254 and JCM7004) contains genes that encode specialised enzymes associated with the extracellular deglycosylation of milk oligosaccharides, including extracellular α-fucosidases [377], β-galactosidases, β-N-acetylglucosaminidases [315] and α-sialidases [356], which efficiently remove monosaccharides from complex milk oligosaccharides. *B. bifidum* and *B. longum* [300] also contain a membrane enzyme, lacto-N-biosidase, responsible for the cleavage of the bifidogenic HMO lacto-n-tetraose to lacto-n-biose [378] and lactose. The mono- and disaccharides released by this endoglycosidase (especially lacto-n-biose), are internalised by family 1 solute binding proteins, and

metabolised. Family 1 solute binding proteins are part of a gene cluster conserved across all infant GIT-associated bifidobacteria, including *B. bifidum*, *B. infantis*, *B. longum* and *B. breve* isolates [370, 379]. The same enzyme degradation mechanism may be responsible for the utilisation of lacto-N-hexaose present in CMO.

None of the selected bifidobacterial strains were able to utilise inulin as the sole carbohydrate source, but all except the *B. bifidum* strains were able to utilise oligofructose. The DP (oligofructose DP 2-10; HP inulin DP 11-60) is likely to influence the ability of bifidobacterial strains to utilise FOS as the sole carbon source [380]. However, bifidobacteria present in breast fed infants may also be selectively stimulated by milk oligosaccharides instead of plant derived oligosaccharides. Previous studies confirmed the poor growth of *B. bifidum* strains on inulin type fructans [381, 382], but strain differences in β-fructofuranosidase production levels have been reported [383]. After weaning, with the introduction of plant derived foods, *B. bifidum* strains are likely to benefit indirectly from the fermentation of inulin type fructans by other members of the GIT microbiota through the lowering of the GIT pH, or the increased availability of monosaccharides as substrates [381].

3.6 Conclusions

In conclusion, bifidobacteria found in faeces (as a proxy for large intestine) of breast fed infants are genetically heterogeneous and, consequently, able to differentially ferment carbohydrates. CMOP was able to stimulate the growth of bifidobacteria commonly found in the GIT of breast fed infants. CMO may also have stimulated the consumption of lactose, glucose, galactose and GOS present in the CMOP. Comparing the selected strains, *B. bifidum* were better able to ferment CMOP, especially the sialyloligosaccharides, which may indicate that *in vivo*, this strain may benefit from CMO consumption. Defining and linking the utilisation of specific oligosaccharide structures, such as the sialyloligosaccharides, to cultured bacteria will provide a scientific path for targeting infant health by establishing protective microbial communities, beneficial to their hosts and potentially applicable to different stages of human life and health states.

Chapter 4 Effects of dietary caprine milk oligosaccharides during pregnancy on mice mono-associated with *Bifidobacterium bifidum* (AGR2166)

Chapter 4

4.1 Introduction

The commensal microbiota of the GIT has important functions for the human host, including development of its function and immune system [384], protection against pathogens [385] and carcinogens [386], nutrient processing [387], stimulation of angiogenesis [388], and regulation of host lipid storage [389]. Early colonisation of the GIT is undoubtedly an important factor for the overall health of the infant [390, 391] and may also have health benefits in later life [130]. Development and initial maturation of the foetal/neonatal GIT occurs partially under maternal control. These effects are thought to be mediated through four main routes: trans-placental transfer of maternal blood factors (e.g. metabolites, proteins, DNA) to the foetus, foetal ingestion of amniotic fluid *in utero*, microbial colonisation of the neonatal GIT by maternal microbiota during, and immediately after birth [114, 169, 392], and maternal milk factors (e.g. oligosaccharides) [161].

Previous literature reported that term foetuses are sterile, and that the initial bacterial colonisation of the neonatal GIT only occurs via contamination by maternal vaginal and faecal bacteria when the neonate moves through the birth channel [93, 393, 394]. It has been demonstrated, however, that the colonisation of the GIT may start *in utero*. The presence of bacteria in amniotic fluid, foetal membranes, umbilical cord blood and infant meconium in healthy mothers [114, 172, 392, 395] has corroborated this hypothesis. The mechanisms of maternal bacterial transfer to the foetus and milk are unclear. It has been proposed that maternal DC and leucocytes play an important role in the bacterial uptake into the placenta and into milk (Figure 1.5) [169]. $CD103^+$ DC in the Peyer's patch, for example, can cross the paracellular space of the GIT epithelium to take up bacteria directly from the lumen [396]. Once internalised by DC and/or macrophages, bacteria can spread to other locations (such as the respiratory and genitourinary tract, salivary and lachrymal glands, and, most significantly, the lactating mammary gland) via the lymphatic and blood circulation

[173, 175, 304]. Once in the blood, maternal derived bacteria may be transferred to the foetus via the paracellular pathway of the placental barrier [178], and breast fed infants receive bacteria derived from maternal milk, which contains up to 10^9 bacteria/L in healthy mothers [397]. These bacteria may originate from the maternal GIT where phagocytes can migrate to lactating mammary glands via lymphatic vessels and blood circulation [169]. There is accumulating evidence that the bacterial composition of the maternal GIT directly affects the microbiota found in foetal membranes and in milk [169, 398]. The period from pregnancy to weaning is likely to be a critical window of opportunity for modifications to the maternal diet for improved infant development and potential long term health benefits.

The addition of prebiotic oligosaccharides to the diet of pregnant mothers has been tested to improve the maternal GIT microbiota population for the benefit of the neonate [27, 228, 246, 399, 400]. The manipulation of human and rodent maternal microbiota through oligosaccharides GOS and FOS, for example, was shown to selectively stimulate the growth and/or activity of beneficial bacteria such as bifidobacteria and lactobacilli; however, those effects were not seen in the offspring [244, 245]. The HMO found in the breast milk may have masked the effects of direct maternal microbiota changes. Another study showed that a diet enriched with GOS and inulin, fed throughout pregnancy and lactation, increased colon length and thigh muscle mass in the offspring [247], suggesting that dietary prebiotics can affect developmental parameters.

HMO are natural oligosaccharides known to stimulate the establishment of the neonatal GIT microbiota by accelerating the development and the maturation of the neonate's GIT (morphologically and immunologically; [401]). Oligosaccharides similar to those found in HMO have been described in caprine milk, with potentially similar beneficial effects for the infant.

GF mice do not have any microorganisms in their GIT (or elsewhere), and provide a tool to investigate how the microbiota, combined with diet, can shape the development of the

immune system and function of the GIT. Mice mono-associated with a specific bacterium can be an efficient tool to show the bacterial translocation route from the maternal GIT to the foetal membranes, or to the foetus. Several morphological and physiological changes are observed during bacterial colonisation of GF rodents (and rabbits), such as increased colon epithelial cell turnover, colon crypt depth, shorter and broader ileal villi [402, 403], thicker colonic epithelial mucus layer and increased numbers of mucus containing goblet cells in the colon [404-406]. Furthermore, it has been shown that bacterial colonisation is associated with changes in the abundance of a number of host genes involved in a variety of functions, including nutrient absorption, metabolism, epithelial barrier function, and maturation of the GIT [116].

Bifidobacteria are health promoting bacteria present in the GIT of breast fed infants, but not commonly found in the GIT of formula fed infants. The study presented in Chapter 3 of this dissertation showed that three bifidobacterial strains (*Bifidobacterium bifidum, Bifidobacterium longum and Bifidobacterium breve*) isolated from infant faecal samples were able to ferment lactose, oligofructose, GOS, 3'-syalyl-lactose or 6'-syalyl-lactose and semi-purified (50%) CMOP (Chapter 2). Of the isolated strains, *B. bifidum* (AGR2166 strain) was the best at fermenting purified CMOP (based on growth and SCFA profiles; Chapter 3), and is likely to be a good candidate to demonstrate the *in vivo* effects of CMOP on the GIT of pregnant mice.

4.2 Hypothesis and Aims

The primary hypothesis of the research presented in this Chapter was that *B. bifidum* AGR2166 and dietary CMO are able to improve colon morphology and the production of SCFA by the colon in pregnant mice. The secondary hypothesis was that *B. bifidum* AGR2166 is able to translocate from the maternal GIT lumen to the foetal membranes.

The first aim was to determine if dietary CMO is able to improve the GIT morphology (i.e. increasing colon crypt length and goblet cells numbers) in pregnant mice mono-associated with the *B. bifidum* AGR2166. The second aim was to determine the translocation of *B. bifidum* AGR2166 in pregnant mice from the GIT lumen to the maternal plasma, spleen, liver, mesenteric lymph nodes (MLN) and foetal membranes.

4.3 Material and methods

4.3.1 Mice

48 GF BALB/c mice (32 female and 16 male) were obtained from Laboratory of Veterinary Public Health, The University of Tokyo between 15 and 23 weeks of age.

4.3.2 Housing

GF mice were housed in a sterile vinyl isolator under otherwise normal conditions (temperature of 22°C, humidity 50 ± 10%, air exchange 12 times/h, 12 h light/dark cycle). This study was approved by the Tokyo University Animal Ethics committee, Tokyo, Japan and performed in accordance with the guidelines for care and use of laboratory animals at The University of Tokyo.

4.3.3 Diet

AIN-76A is a commonly used research diet, formulated to meet the requirements of adult mice and rats using bovine casein as the protein source (78). Two different diets, based on AIN-76A (AIN-76A supplemented with CMOP containing 1% CMO (CMO diet), and AIN-76A supplemented with GOS, lactose, glucose and galactose (combo diet)), were formulated by Research Diets, Inc. (USA) to meet mouse nutritional requirements (Table 4.1) and made by Oriental Yeast Co. Ltd. (Japan). The combo diet contained sugars (GOS, lactose, glucose and galactose) at the same concentration that are present in the CMO-enriched fraction (Table 4.2). Maltodextrin concentration was adjusted in the CMO and combo modified diets

Table 4.1 Combo and caprine milk oligosaccharide diet composition based on AIN-76A diet

Ingredient (%)	Combo	CMO
Protein	20	20
Carbohydrate	67	67
Fat	5	5
Diet composition (g/Kg)		
Casein	200	200
Corn oil	50	50
Cellulose, BW200	50	50
DL-Methionine	3	3
Mineral Mix S10001	35	35
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Corn Starch	500	500
Maltodextrin	128.1	118.1
Glucose (dextrose monohydrate)	10	10
Galactose	6.6	6.6
Lactose	3.3	3.3
Galacto-oligosaccharides	2	2
Caprine milk oligosaccharides	0	10

for energy and nutritional balance. All ingredients of the combo and CMO diets except CMOP (sourced from New Zealand) and GOS (sourced from Yakult - Japan) were supplied by Oriental Yeast Co. Ltd. (Japan). Diets were stored at room temperature in a cool dry place until fed.

4.3.4 Study design

The experiment was conducted using a 2x2 factorial design. Control mice (control) or mice mono-associated with *B. bifidum* AGR2166 (Bifidobacteria) were fed either combo or CMO diet (Figure 4.1). On day one of the experimental period, mice were randomly assigned to groups of six (four females and two males), ensuring equal age distribution among treatment groups (Figure 4.2), and fed either combo or CMO diet.

The *B. bifidum* AGR2166 were cultured on BL agar plates (a selective medium for *Bifidobacterium* spp. [407]) anaerobically for 2 days. A few colonies grown on the BL agar plates, were picked and cultured on new BL agar plates for one day prior to inoculation into GF pregnant mice. After cultivation, one loop-full of the colonies were suspended in anaerobic phosphate buffer solution (anaerobic phosphate buffer solution (PBS): 4.5 g KH_2PO_4, 6.0 g $NaHPO_4$, 0.5 g L-cysteine hydrochloride, 0.5 g Tween 80, and 0.75 g agar in 1 L distilled water). The GF female and male were inoculated with *B. bifidum* AGR2166, 0.5 mL of bacterial suspension containing approximately 10^8 CFU/mL of viable bacteria, or a 0.5 mL of anaerobic phosphate buffer solution (GF control) via oral gavage.

Females were examined each morning to define the phase of their oestrous cycle and to detect the presence of a sperm plug to confirm pregnancy. On day 7 of the experimental period, males were removed and humanely euthanised. Due to the difficulty of evaluating the pregnancy status in control mice, all females were kept until the predicted due date or last day of sampling.

Table 4.2 Composition of caprine milk oligosaccharide enriched product added to the experimental AIN-76A based diet

Nutrient	Units	Quantity
Protein	(g/kg)	0.4
Fat	(g/kg)	22
GOS	(g/kg)	59.8
Lactose	(g/kg)	100.0
Glucose	(g/kg)	299.4
Galactose	(g/kg)	200.0
Oligosaccharides	(g/kg)	299.4
Calcium	(g/kg)	0.8
Magnesium	(g/kg)	0.4
Potassium	(g/kg)	11.0
Sodium	(g/kg)	6.2
Iodine	(mg/kg)	0.002
Selenium	(g/kg)	<0.003

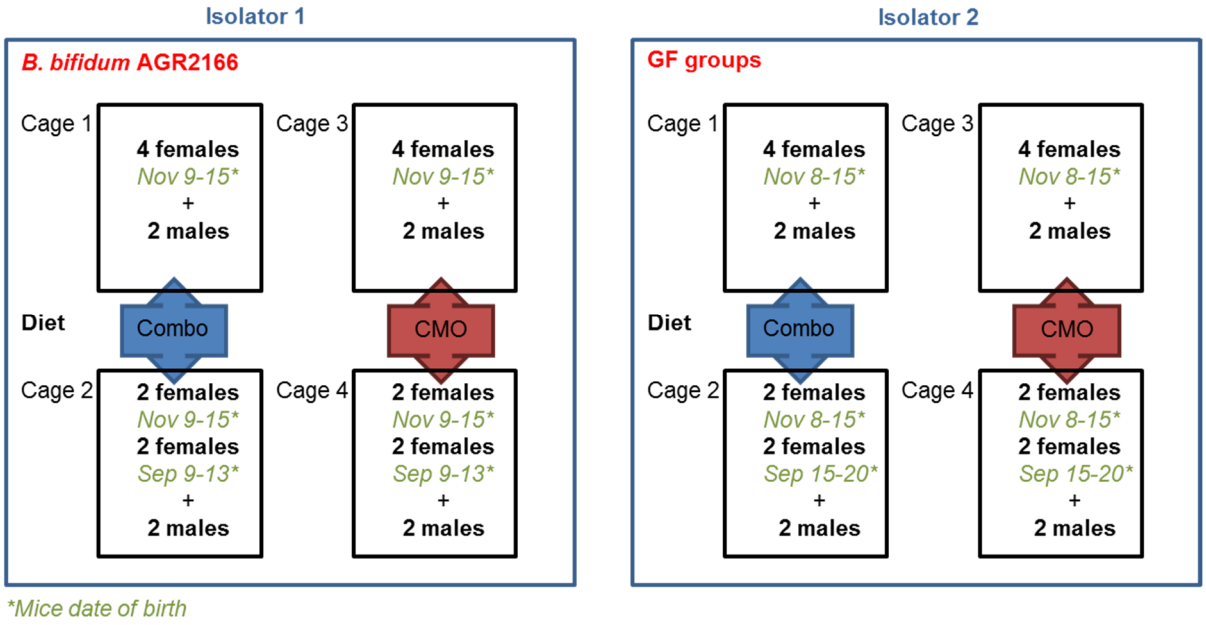


Figure 4.1 Distribution of mice by age and sex in the germ-free isolators.

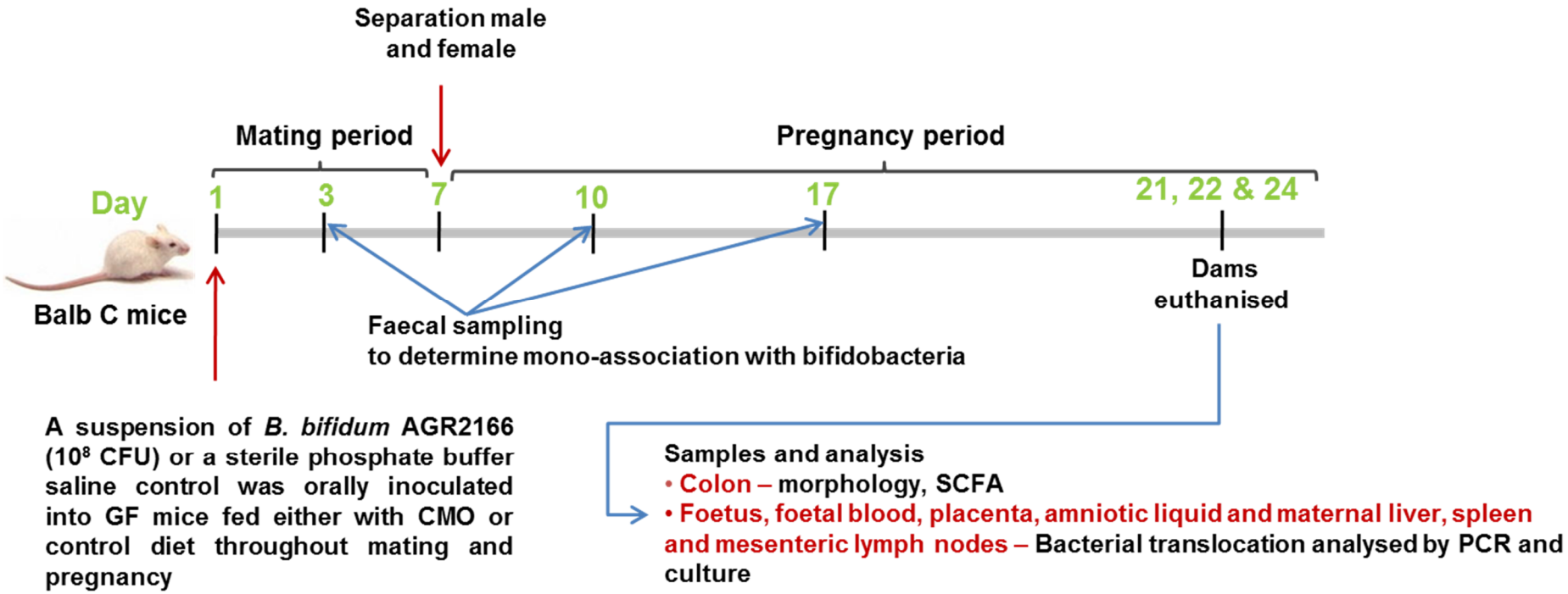


Figure 4.2 Study design

4.4 Sampling

4.4.1 Faecal sampling

The ability of the *B. bifidum* AGR2166 to colonise the GIT of GF pregnant mice were evaluated using fresh faecal samples collected on days 3, 10 and 17 of the experimental period. Faecal samples were aseptically collected from mice inside the isolators into a sterile tube and kept on ice. Samples were immediately processed and cultured.

4.4.2 Tissue samples

At the end of the experimental period (1 to 3 days before the expected delivery date), mice were euthanised by carbon dioxide anaesthesia followed by cervical dislocation. Immediately before sampling, mice were fasted for 14 h overnight, re-fed for 2 h and fasted again for 2 h to minimise variation in food intake and its affects on the parameters analysed [408]. Each mouse was sampled over approximately 15 min so the fasting and re-feeding was staggered by one hour in groups of 4 to 5 mice.

Maternal blood was aseptically collected by cardiac puncture and approximately half the volume was stored at -80°C until DNA extraction, and half plated on a BL agar plate. Plates were anaerobically incubated at 37°C and *B. bifidum* growth was assessed after 48 h. Each uterus was removed intact from the abdominal cavity, weighed and amniotic fluid aseptically collected by puncturing each placenta with a sterile syringe. Each foetus was removed from the uterus, counted and one foetus collected for DNA extraction to investigate the presence of *B. bifidum* AGR2166. Foetus blood was collected from the remaining foetuses by decapitation. Blood was collected into an EDTA coated sterile capillary tube, and approximately half the volume plated on BL agar plates, and half the volume snap frozen for DNA extraction to determine the presence or absence of *B. bifidum* AGR2166.

To avoid contamination, animals were transferred to a laminar flow cabinet for sampling after euthanasia. All sampling material was changed or sterilised between animals. Each maternal organ to be examined (spleen, liver, MLN, digesta and intestinal tissue) was divided into two, with one sample snap frozen for further DNA extraction and the other sample homogenised in a sterile glass homogeniser containing sterile anaerobic PBS. This homogenate was then plated on BL agar plates, anaerobically incubated at 37°C for 48 h, and *B. bifidum* colonies counted.

The GIT was removed, and the length to weight ratio recorded prior to full dissection. The caecum was dissected, and the contents collected into a labelled tube and snap frozen for SCFA analysis. Transverse sections (proximal section) of the colon were collected and stored in 10% formalin solution (1 mL per sample) at room temperature for subsequent histological assessment of the colonic crypt length and goblet cells numbers.

4.5 Sample analyses

4.5.1 Diet composition

Dietary oligosaccharide composition was analysed by LC-MS, using a graphite column [271, 409] on a Thermo Scientific LTQ XL-Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionisation in negative mode. Data were collected in profile data acquisition mode over the mass range 300 to 2000 mass/charge (m/z) and processed using the Xcalibur software package provided by the manufacturer. Oligosaccharides were identified and quantified based on calibration curves of purified standards prepared in water ranging from 0.1 to 0.001 mg/mL; 4-galactosyllactose (Glentham Life Sciences, UK), 3'- and 6'-sialyl-lactose milk oligosaccharide; 3- and 2-fucosyllactose, lacto-N-hexaose, disialyllactose (Carbosynth, UK). While the various oligosaccharides have different ionisation efficiencies during MS analysis, the lack of purified oligosaccharide calibration standards available meant that, for this study, where there were

no oligosaccharides standards available, quantification was estimated using the response curves of the purified 3'-sialyl-lactose.

Lactose, glucose, galactose and GOS concentrations were analysed by high pressure ion chromatography (HPIC) using a DIONEX ICS-5000+ SP/DC (Thermo Fisher Scientific, Waltham, MA, USA). HPIC is more sensitive than HPLC methods for carbohydrates. A CarboPac PA20 3x30 mm column was used at 30°C and a flow rate of 0.5 mL/min, with an ED50 electrochemical detector. The Dionex CarboPac PA20 is a strong anion exchange column providing high resolution separations of mono- and disaccharides with no need for derivatisation [410]. A 10 µL sample was injected and run, followed by a regeneration step and an equilibration. Data were collected using the Chromeleon Chromatography Data System software (V7.2.0.4154). Lactose (BDH), glucose (BDH), galactose (BDH) and GOS (kindly donated by Yakult, Japan) standards were prepared in water ranging from 0.2 to 0.45 g/mL. Diet pellets for both LC-MS and HPIC analysis, were dissolved in water to a final concentration of 1 g/mL, centrifuged for 1 min at 10,000 x g to remove excess solids, and filtered using 0.22 µm filters (Millore Australia Pty Ltd, NSW, Australia).

4.5.2 Bifidobacterial population in faeces

Faecal pellets were weighed and transferred to an anaerobic cabinet. Faecal samples were homogenised using a sterile glass pestle (5 mL) (Corning Pirex, Tokyo, Japan) with 50 volumes of anaerobic PBS, and serial tenfold dilutions were plated onto BL agar plates (0.1 mL of the dilutions 10^{-6} and 10^{-7}). After anaerobic incubation at 37°C for 96 h, colonies were counted and collected for identification. The gram staining method was used to identify colony phenotype and to evaluate contamination.

4.5.3 Histology

Formalin fixed transverse, paraffin embedded sections of the colon were stained with haematoxylin and eosin. Morphology measurements were performed using bright field

microscopy at 200 times magnification and Image-Pro Plus 4.0 (MediaCybernetics, Bethesda, MD, USA). Crypt lengths were determined by measuring an average of 80 random fully longitudinally sectioned crypts from the base of the crypt to the flat margin of the colon mucosa in all three colon sections for each mouse. Similarly, numbers of goblet cells per crypt were determined by counting goblet cells in an average of 30 random fully longitudinally sectioned crypts per mouse.

4.5.4 Short chain fatty acids composition in the caecum

Caecum content samples were thawed and combined with the homogenisation medium PBS. The digesta were homogenised for 30 s using an Ultra-Turrax homogeniser at maximum speed (IKA, Germany) and centrifuged at 2800 g at 4°C for 15 min to remove particulate material. The supernatant was then collected and filtered using 0.22 µm filters (Millipore Australia Pty Ltd, NSW, Australia). The filtered supernatants were analysed for SCFA, lactic acid, succinic acid and glucose concentrations using a Shimadzu RID 10A HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand) fitted with a Bio-Rad Aminex 87H 9µm, 7.5 x 300 mm HPLC column kept at 45°C. The mobile phase was sulphuric acid (5 mM) using an isocratic elution with a flow rate of 0.8 mL/min. The injected volume was 50 µL and the run time was 35 min between injections. The SCFA concentrations in the samples were quantified on the basis of known amounts of standards (Shimadzu Oceania, Pickering Laboratories, USA), using LC Solution ver. 1.22 SP1 software (Shimadzu, Kyoto, Japan). Identification and quantification of peaks were performed by running single standards of acetic acid, propionic acid, ethyl-butyric acid, butyric acid, isovaleric acid (3-methylbutanoic acid), valeric acid (pentanoic acid), formic acid, lactic acid, succinate and glucose.

4.5.5 Isolation of bifidobacteria DNA from maternal tissue and caecum content

Prior to bacterial DNA extraction, organ samples and foetuses were transferred to a laminar flow cabinet and homogenised using a hand held homogeniser (OMNI international,

Kennesaw, USA) at full speed for 30 s in sterile PBS (50 to 200 µL). Total DNA was extracted from maternal plasma, MLN, spleen, liver, placenta, amniotic fluid, foetus and caecum content using DNeasy Blood & Tissue Kit (Qiagen, Biolab NZ). Samples were pre-treated, using lysis buffer (20 mM Tris·Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton® X-100 and lysozyme to 20 mg/ml) and proteinase K from the DNeasy kit, and incubated for 30 min at 37°C and 56°C for 30 min respectively, according to manufactures instructions for extraction of gram positive bacterial DNA. Samples were boiled for 1 min or transferred to a bead beater tube containing 0.3 g of sterile 0.1 mm diameter zirconium beads (Sigma-Aldrich) and shaken in a mini bead beater (Biospec Products, Bartlesville, OK, USA) set at maximum for 45 s, followed by centrifugation at 13,000 X g for 1 min before being added to the DNeasy Mini spin column.

From the isolated genomic DNA, the bifidobacteria 16S rRNA gene was amplified using bif 164 (5′ GGG TGG TAA TGC CGG ATG 3′) and bif 662 (5′ CCA CCG TTA CAC CGG GAA 3′) primers [343]. For PCR, 25 µL reactions were made up containing 0.5 µL of each primer (stock 10 pM μL^{-1}), 2.5 µL PCR buffer (10X), 0.75 µL $MgCl_2$ (50mM), 0.5 µL dNTP mixture (0.2 mM each dNTP), 0.1 µL Platinum Taq DNA Polymerase (Invitrogen) and 1 µL DNA template (15 ng μL^{-1}). Amplification was done using a PX2 Thermal Cycler (Ashford, UK) with an initial denaturation cycle of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 45 s, and extension at 72°C for 45 s and with a final extension at 72°C for 10 min. Verification of successful amplification was checked using agarose gel electrophoresis of PCR products. The presence of bacterial contamination in maternal plasma and liver were tested by PCR amplification of the bacterial 16S rDNA using the universal bacterial primers fD1, 5′-AGA GTT TGA TCC TGG CTC AG-3′ and rD1, 5′-AAG GAG GTG ATC CAG CC-3′ [411].

4.6 Statistical analysis

Statistical analysis was performed in GenStat (version 15; VSN international Ltd., Hemel Hempstead, Hertfordshire, UK). Due to unequal sample size, treatments were compared using an unbalanced ANOVA. Although it was a small sample set, samples were still tested for normality and homogeneity of variances and shown to be acceptable. The difference between pregnancy rate and bifidobacteria translocation between the treatments were evaluated by binomial test. Statistical difference was declared between two treatments when the difference in means was greater than the least significant difference (LSD) at 5%.

Samples from inoculated and control mice were identified using the term Bifidobacteria and Control respectively. Samples from mice fed combo and CMO containing diet were identified using the terms combo and CMO respectively.

4.7 Results

4.7.1 Changes in diet composition

Formulated diets were analysed in order to confirm that the macronutrient composition was as calculated for the test components (Table 4.3). Lactose, glucose and galactose were present in different concentrations than expected in combo and CMO diet. Combo diet had double the amount of lactose (combo, 2.4 g/kg; CMO, 1.1 g/kg) and approximately half the amount of glucose (combo, 5.5 g/kg; CMO, 9.8 g/kg) and galactose (combo, 3.4 g/kg; CMO, 8.7 g/kg) compared to CMO diet. The CMO concentration in the CMO diet was also 10% lower than expected.

4.7.2 Dams and litters

Of the eight female mice originally mated in each treatment group, only one and three females in control mice fed combo and CMO diet respectively were pregnant by the end of the seven days of mating. The seminal plug was visible in 17 out of 24 mated females and

Table 4.3 Carbohydrate composition of diets as tested at the end of the experiment. Expected values from the diet formulation are shown in parenthesis.

Test components (g/kg)	Combo	CMO
GOS	1.8 (2.0)	2.0 (2.0)
Lactose	2.4 (3.3)	1.1 (3.3)
Glucose	5.5 (10.0)	9.8 (10)
Galactose	3.4 (6.6)	8.7 (6.6)
CMO	0 (0)	9 (10)

predicted the delivery date of all 11 dams within one to three days of the due date. Mice were sampled within one to three days of the due date, therefore no comparison between dams, foetal and uterus weights was possible. Due to the low mating success in the control mice fed combo diet (n = 1), no comparisons were drawn between control mice fed combo diet group and the other treatments.

4.7.3 Bifidobacterial population in mouse intestine

Bifidobacteria numbers recovered from faeces remained stable during the three collection days (10^8 CFU per gram of faeces). No significant differences were found between treatments and sampling days (Figure 4.3). The presence of *B. bifidum* in the caecum of inoculated mice was also confirmed by the successful amplification of the *B. bifidum* 16s rRNA gene (Figure 4.4), and no amplification of the gene was observed in digesta from control mice (Figure 4.4).

4.7.4 Body and organs weights

It was expected that there would be a significant effect of maternal body weight on foetus and organs weight, so all organ weights and lengths are expressed as a proportion of individual body weight (normalised data).

There was no evidence that diet, bifidobacteria or the interaction between diet and bifidobacteria inoculation affected body weight, uterus, spleen, liver and MLN weight and normalised organ weight (Table 4.4). However, the number of foetuses were higher in mice mono-associated with bifidobacteria when compared to the control mice (Control, 5.0 ± 1.32; Bifidobacteria, 6.6 ± 0.6; mean ± SE, P = 0.03) (Table 4.5). Foetus weight was also significantly increased in mice mono-associated with bifidobacteria (Control, 3578; Bifidobacteria, 5308 ± 818; mean (mg) ± SE, P = 0.04). Dams fed the combo diet had a higher relative foetus weight (Combo, 131; CMO, 98 ± 18; mean (mg/g) ± SE, P = 0.03) compared to CMO fed dams (Table 4.6). Mice mono-associated with

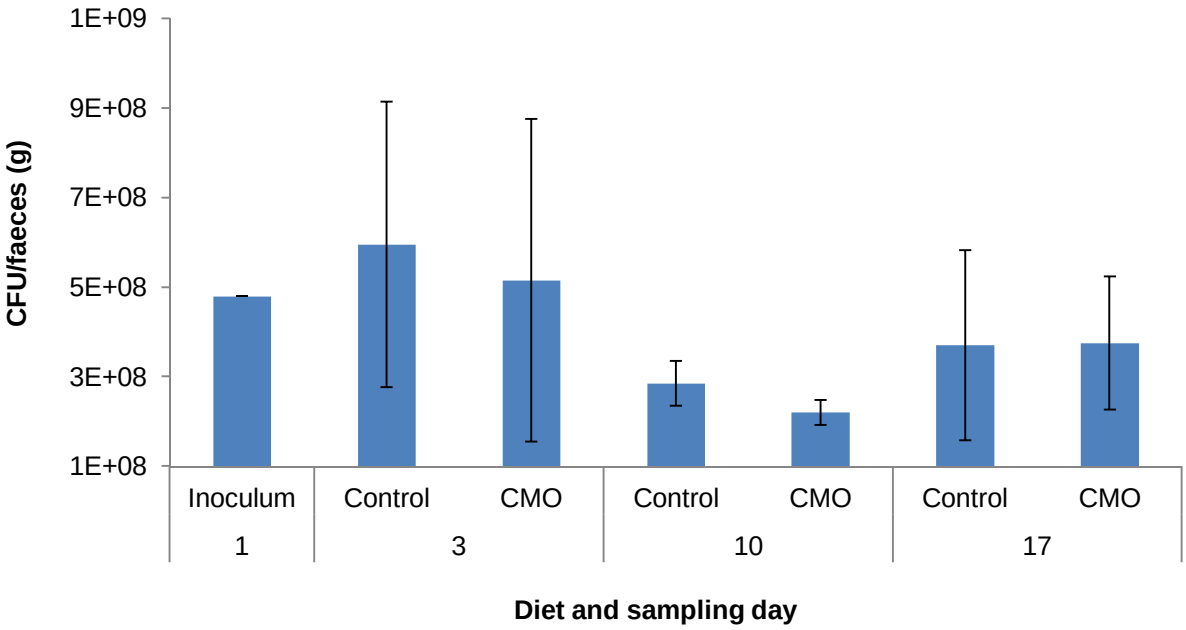


Figure 4.3 Population stability of *Bifidobacterium bifidum* AGR2166 at day 1, 3, 10 and 17 of the experimental period. Data are viable counts of bifidobacteria obtained from faecal pellets. Each time point is an average of faecal pellets collected from three different animals and the errors bars indicate standard deviation.

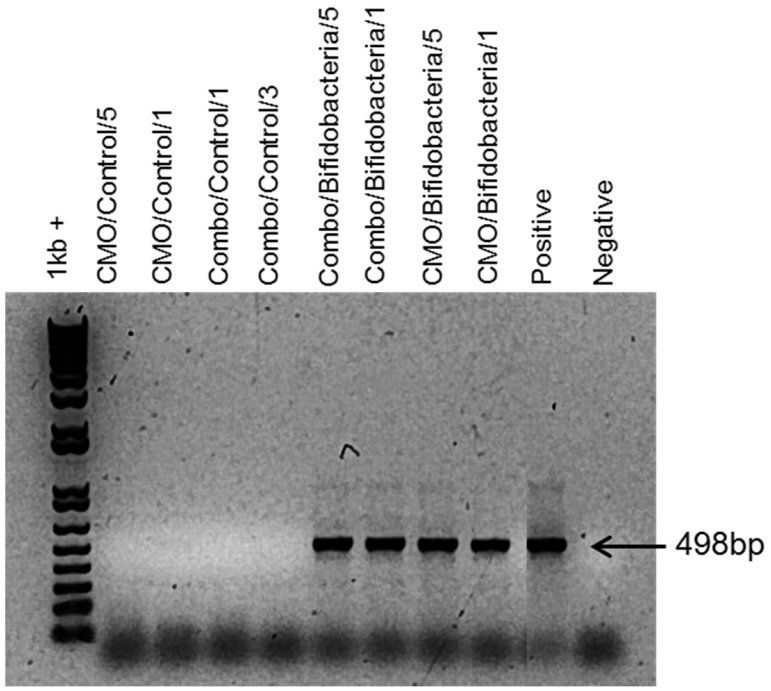


Figure 4.4 *Bifidobacterium bifidum* AGR2166 DNA extracted and amplified from maternal caecal contents. PCR using specific bifidobacteria primers bif662 and bif164. Samples named according to diet, caprine milk oligosaccharide (CMO) or combo; bifidobacteria inoculation (Control, control; Bifidobacteria, inoculated with *B. bifidum*) and animal number (1, 3 or 5). Ladder 1kb plus (Life Technologies). Caecum samples obtained from mice inoculated with bifidobacteria should show the amplification of a 498 bp region of the 16S rRNA gene corresponding to the V2 to V3 variable regions.

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Table 4.4 Absolute and normalised mean body weight, organ weight and gastrointestinal tract length in dams among treatment groups

Treatments*	Control/Combo	Control/CMO	Bifidobacteria/Combo	Bifidobacteria/CMO	SE	P-value	LSD
Body weight (g)	38.5	40.8	40.8	43.7	2.6	0.8	6.2
Absolute organ length and weight							
Uterus weight (mg)	5740	6513	8302	7656	1272	0.4	3007
Number of foetus	5.0	5.0	6.2	7.0	0.9	0.5	2.2
Foetus weight (mg)	3280	3827	6413	4387	1166	0.1	2757
Spleen weight (mg)	81	84	84	86	9	0.9	22.1
Liver weight (mg)	169	179	212	197	28	0.5	67
MLN weight (mg)	18	31	20	28	9	0.6	21.6
GIT length (cm)	55	56	51	59	4	0.3	10
GIT weight (mg)	5819	5860	4474	5774	621	0.2	1470
Relative organ length and weight per mouse body weight							
Uterus weight (mg/g)	148	161	203	174	32	0.4	77
Foetus weight (mg/g)	85	94	157	100	28	0.1	67
Spleen weight (mg/g)	2.1	2.1	2.0	1.9	0.2	0.8	0.4
Liver weight (mg/g)	4.3	4.4	5.1	4.5	0.6	0.5	1.5
MLN weight (mg/g)	0.4	0.7	0.5	0.6	0.2	0.6	0.5
GIT length (cm/g)	1.4	1.3	1.2	1.3	0.07	0.2	0.1
GIT weight (mg/g)	150	143	109	131	11	0.1	28

*Treatments are identified as Control and Bifidobacteria for non-inoculated and inoculated mice respectively. Combo and CMO containing diet were identified as combo and CMO, respectively (n= Control/Combo, 1; Control/CMO, 3; Bifidobacteria/Combo, 4; Bifidobacteria/CMO, 3).

Table 4.5 Effect of bifidobacteria on dam absolute and normalised mean body weight, organ weight and gastrointestinal tract length

Treatments*	Control	Bifidobacteria	SE	P-value	LSD
Body weight (g)	39.8	42.4	1.8	0.1	4.4
Absolute organ length and weight					
Uterus weight (mg)	6162	7950	893	0.07	2011
Number of foetus	5.0	6.6	0.6	0.03	1.5
Foetus weight (mg)	3578	5308	818	0.04	1936
Spleen weight (mg)	83	85	6	0.7	15
Liver weight (mg)	174	204	20	0.1	47
MLN weight (mg)	25.2	24.6	6.4	0.4	15.2
GIT length (cm)	55.0	55.4	3.1	0.6	7.3
GIT weight (mg)	5842	5183	436	0.08	1033
Relative organ length and weight per mouse body weight					
Uterus weight (mg/g)	155	188	23	0.2	54
Foetus weight (mg/g)	90	126	20	0.1	47
Spleen weight (mg/g)	2.08	2.02	0.1	0.6	0.3
Liver weight (mg/g)	4.3	4.8	0.4	0.4	1.1
MLN weight (mg/g)	0.6	0.5	0.1	0.6	0.3
GIT length (cm/g)	1.39	1.30	0.05	0.1	0.1
GIT weight (mg/g)	147	121	8	0.02	19

* Treatments are identified as Control and Bifidobacteria for non-inoculated and inoculated mice respectively (n= Bifidobacteria, 7; Control, 4)

Table 4.6 Effect of diet on dam absolute and normalised mean body weight, organ weight and gastrointestinal tract length

Treatments*	Combo	CMO	SE	P-value	LSD
Body weight (g)	40.0	42.7	1.7	0.3	4.2
Absolute organ length and weight					
Uterus weight (mg)	7371	7241	849	0.8	2008
Number of foetus	5.7	6.2	0.6	0.4	1.4
Foetus weight (mg)	5273	4183	778	0.1	1841
Spleen weight (mg)	83	85	6	0.6	14
Liver weight (mg)	196	191	19	0.7	45
MLN weight (mg)	19	29	6	0.1	14
GIT length (cm)	52	57	3	0.09	7
GIT weight (mg)	4963	5806	415	0.06	981
Relative organ length and weight per mouse body weight					
Uterus weight (mg/g)	183	170	22	0.2	52
Foetus weight (mg/g)	131	98	19	0.03	44
Spleen weight (mg/g)	2	2	0.1	0.7	0.3
Liver weight (mg/g)	4.8	4.4	0.4	0.2	1.0
MLN weight (mg/g)	0.4	0.6	0.1	0.1	0.3
GIT length (cm/g)	1.31	1.35	0.04	0.1	0.1
GIT weight (mg/g)	124	136	8	0.03	18

* Combo and CMO containing diet were identified as combo and CMO, respectively (n= Combo, 5; CMO, 6)

bifidobacteria had a significant lower relative GIT weight (Control, 147; Bifidobacteria, 121 ± 8; mean (mg/g) ± SE, P = 0.02). CMO diet tended to increase GIT weight (Combo, 4963; CMO, 5860 ± 415; mean (mg) ± SE, P = 0.06) and relative GIT weight (Combo, 124; CMO, 136 ± 8; mean (mg/g) ± SE, P = 0.03) compared to the combo diet (Table 4.6). Since there was only one observation for control mice fed the combo diet treatment, the predicted mean for this combination would be the observation itself, and would not be reliable as a mean. No variation for this treatment could be estimated. Also the effects of diet and bifidobacteria interaction may have been influenced by this observation.

4.7.5 Histology

There was no evidence that the interaction between diet and bifidobacteria affected the number of goblets cells and the size of crypts of the dams (Table 4.7). However, there was a trend (defined as P > 0.05 but < 0.1) for an increase in the number of goblet cells (Control, 19.1; Bifidobacteria, 22.1 ± 2.1, mean ± SE, P = 0.09) and crypt size (Control, 172.6; Bifidobacteria, 183.9 ± 8.7, mean (µm) ± SE, P = 0.1) in inoculated dams. There was also a trend for the number of goblet cells in dams fed the combo diet to be higher (combo, 22.2; CMO, 20.9 ± 2.0, mean ± SE, P = 0.1), regardless of the inoculation status.

4.7.6 Caecal short chain fatty acids

Acetic acid, ethyl butyric acid, isovaleric acid and valeric acid were not detected in the dam caecum digesta. Succinic, lactic, formic and propionic acids were detected, but were not uniformly distributed between the treatments (only detected in a few samples in some treatments; (Table 4.8). Formic acid, for example was only detected in dams fed the combo diet and inoculated with bifidobacteria. Propionic acid was only detected at low concentration (3.7 µmol/g) in one sample from control mouse fed combo diet. Lactic acid and glucose were detected mainly in dams inoculated with bifidobacteria. Succinic acid was increased

Table 4.7 Goblet cells number and colon crypt length in control mice and mice inoculated with bifidobacteria fed either combo or oligosaccharides diet

*Treatments	Control/Combo	Control/CMO	Bifidobacteria/Combo	Bifidobacteria/CMO	SE	P-value	LSD
Goblet cells	20.8	17.7	23.1	21.4	3.0	0.7	7.2
Crypt size	177.3	168.8	185.9	182.2	12.4	0.8	29.5

*Treatments are identified as Control and Bifidobacteria for non-inoculated and inoculated mice respectively. Combo and CMO containing diet were identified as combo and CMO, respectively (n= Control/Combo, 1; Control/CMO, 3; Bifidobacteria/Combo, 4; Bifidobacteria/CMO, 3).

Table 4.8 Glucose and short chain fatty acids concentrations (μmol/g colon digesta) in control and inoculated dams fed either combo or caprine milk oligosaccharide diet

Animal ID	Glucose	Succinic	Lactic	Formic	Propionic
Control/Combo/1	nd	nd	nd	nd	nd
Control/ CMO/1	nd	142.9	nd	nd	nd
Control/ CMO /2	nd	237.3	nd	nd	nd
Control/ CMO /3	3.5	109.9	3.9	nd	nd
Bifidobacteria/ Combo/1	14.2	4.3	161.6	192.1	nd
Bifidobacteria/ Combo/2	15.0	6.8	333.4	130.6	3.7
Bifidobacteria/ Combo/3	11.7	3.6	255.6	188.3	nd
Bifidobacteria/ Combo/4	6.8	0.3	348.0	197.1	nd
Bifidobacteria/ CMO/1	9.1	5.5	128.6	nd	nd
Bifidobacteria/ CMO/2	nd	30.9	122.9	nd	nd
Bifidobacteria/ CMO/3	nd	29.1	164.4	nd	nd

*Animals are identified as Bifidobacteria and Control for inoculated and control mice respectively. Combo and caprine milk oligosaccharide diet were identified as combo and CMO respectively.

nd, not detected. Limit of detection varied with the short chain fatty acids measured.

in the caecum of inoculated animals fed combo compared to CMO diet (Bifidobacteria/combo, 274.7, Bifidobacteria/CMO, 138.7 ± 51.7, mean (µmol/g) ± SE, P = 0.04). Lactic acid was not detected in control mice fed combo or CMO diet. Control mice fed CMO diet, however, had higher concentrations of succinic acid compared to inoculated mice regardless of the diet (Control/CMO, 163.4, Bifidobacteria/combo, 3.8, Bifidobacteria/CMO, 21.9 ± 34.8, mean (µmol/g) ± SE, P = 0.02). Among inoculated mice, diet had no significant effects on caecal succinic acid concentrations.

4.7.7 Bifidobacterial translocation to maternal tissue

The ability of *B. bifidum* AGR2166 to translocate alive from maternal GIT to the maternal organs, pregnancy membranes and foetus were tested by anaerobically culturing the samples on BL plates. No growth of the bacterium was detected after 48 h. However, the presence of *B. bifidum* AGR2166 DNA in some of the maternal organs and placenta was detected by amplification of the bifidobacterial 16S rRNA gene in all treatment groups (Table 4.9). Bifidobacteria DNA was detected in the MLN, liver, plasma and placenta of inoculated pregnant mice. No amplification of the bifidobacterial 16S rRNA were detected in the spleen, foetus and amniotic fluid samples. In animals that received the CMO diet, bifidobacteria DNA was detected in all the organs tested and in a greater number of samples when compared to combo fed animals (combo, 7 out of 20; CMO, 12 out of 15 samples tested, P = 0.08). Bifidobacteria DNA was detected in almost all placenta samples tested in inoculated animals (6 out of 7 samples). Bifidobacteria were also detected in few samples of control dams (1 MLN, 2 liver and 1 plasma samples) in both diets. Bifidobacteria from inoculated dams samples was the likely source of contamination since the homogenisation and DNA extraction of all samples were done in one batch.

Table 4.9 Amplification of 16S rRNA gene of *B. bifidum* AGR2166 from DNA extractions of organs taken from dams post mortem

Treatments*	**Bifidobacteria/Combo**				**Bifidobacteria/CMO**			**Control/Combo**	**Control/CMO**		
Animal#	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**1**	**1**	**2**	**3**
MLN						X	X				X
Liver		X			X		X	X		X	
Plasma	X		X		X	X	X	X			
Placenta	X		X	X	X	X	X				

*Treatments are identified as Bifidobacteria and Control for inoculated and control mice respectively. Combo and caprine milk oligosaccharide diet were identified as combo and CMO respectively.

Organs where the *B. bifidum* 16S rRNA gene was detected are marked (X).

4.8 Discussion

This study investigated the effects of dietary CMO in the colon crypt length, goblet cell numbers and SCFA production of pregnant mice mono-associated with the *B. bifidum* AGR2166. CMO-fed dams inoculated with *B. bifidum* AGR2166 showed no effects on bacterial fermentation and morphology in the colon. *B. bifidum* AG2166, however. independent of diet; decreased GIT weight and increased bacterial fermentation. These results disproved the first hypothesis which stated that the combined effects of *B. bifidum* AGR2166 and dietary CMO would improve the morphology and the production of SCFA in the colon of pregnant mice. *B. bifidum* AGR2166 DNA was detected in the maternal organs and placenta, however *B. bifidum* AGR2166 was not able to be cultured from these organs. Although *B. bifidum* AGR2166 could not be cultured, the presence of bacterial DNA on maternal organs could be an indication of bacterial translocation. Thus, no conclusions were obtained in regard to hypothesis two.

4.8.1 Fecundity and foetus growth

Low levels of fecundity were observed in all treatments, restricting the number of samples analysed in this study. Low pregnancy rate and small litter size are reported limitations for the use of GF mice, due to physiological deviations from the conventional model [338, 412]. Prolonged diestrus in the estrus cycles and subsequent decrease in the frequency of estrus; sperm motility, copulation rate, implantation rate [413] and decreased progesterone values [414, 415] have been reported in GF mice. The presence of bifidobacteria in the GIT of GF females and GF males, during the experimental period did not improve fecundity. However, GF mice inoculated with bifidobacteria, showed an increased number and weight of foetuses. Increase in foetal weight was also observed in dams fed the combo diet.

Foetal weight is influenced and regulated by nutrients supplied by the mother [416, 417]. It also depends on the maternal body composition and size, nutrient stores, diet during

pregnancy, nutrient absorption, and nutrient transport to and across the placenta [417]. Food digestion and nutrient absorption in GF mice is influenced by morphological modifications in the GIT as a consequence of the absence of microbiota [418]. Caecal enlargement [419], reduction in mucus producing goblets cells, crypt depth and villus height in the SI, for example, are features of GF mice [411, 418, 420, 421]. GF mice require 30% more energy than conventionally raised mice to maintain the same body weight [412], and this may influence the nutrients available for foetal growth. Bacterial colonisation of GF mice can revert some of the physiological characteristics from GF to a similar state to conventional mice and improve nutrient absorption [403].

4.8.2 Gastrointestinal weight and short chain fatty acids

Oral administration of *B. bifidum* AGR2166 to GF mice, independent of the diet, was shown to decrease the GIT weight although a trend for increased crypt size and goblet cell number was observed. Bacterial colonisation of GF mice, however, is generally characterised by thickening of the lamina propria associated primarily with cellularity [422], thickening of the submucosa (containing large blood vessels, lymphatics, and neural complexes), increased proportion of acidic mucins in the luminal content [423] and increased muscular layers [424]. This paradoxical response (i.e. the decrease in the GIT and increased crypt size and goblet cell number) may be the result of villus lengthening in GF mice compared with the mono-associated mice [403]. *B. bifidum* colonisation may also have decreased caecal enlargement, reflected by the GIT weight.

The consumption of CMO, regardless of bifidobacterial inoculation, was shown to increase GIT weight. Dietary fibres [425-429], and even other indigestible substrates [430], have been shown to increase mucin secretion in the SI and colon as a consequence of both SCFA production [425] and mechanical stimulation by increased faecal mass [429] [428]. Increase in mucin production and increased faecal mass may have contributed to increase GIT weight.

A higher concentration of succinic acid, was detected in the GF mice fed CMO compared to inoculated mice. Trace amounts of SCFA have previously been reported in GF mice [431], and it is likely that these arise from cellular metabolism in the mice as a result of the oligosaccharides in the CMO diet. In addition, bifidobacteria was shown to produce millimolar concentrations of succinic acid while actively growing, ceasing when nutrients are depleted [432]. SCFA were found more frequently in inoculated mice regardless of the diet compared to non-inoculated. SCFA are the main end products of anaerobic bacterial metabolism in the GIT [433]. Like other *Bifidobacterium* species, *B. bifidum* is a lactic acid bacterium that has been reported to produce lactic acid, acetic acid, ethanol, and/ or formic acid, depending on the carbohydrate subtract used [373, 434, 435]. Lactic and acetic acids are the principal SCFA produced by *Bifidobacterium* species on glucose substrate [434].

In contrast to Chapter 3, where *B. bifidum* AGR2166 produced acetic and lactic acid *in vitro*, it was observed that *B. bifidum* preferentially produced lactic acid and formic acid; the latter only detected in combo fed mice indicating a contrasting end product of bifidobacteria fermentation. Van der Meulen *et al* [432] reported that where the bifidobacteria growth rate increases, the end products favour higher concentrations of lactic acid and lower concentrations of formic acid, acetic acid and ethanol. Lactic acid is produced during glucose fermentation through the reduction of pyruvate by the enzyme lactate dehydrogenase (EC 1.1.1.27) (fructose-6-phosphate phosphoketolase pathway) [436]. Lactose, highly concentrated in the combo compared to CMO diet, is likely to be degraded to galactose and glucose in a catabolic reaction that requires β-galactosidase activity. *B. bifidum* contains both extracellular and intracellular β-galactosidases [374], which may explain the higher concentration of glucose found in inoculated mice. A high concentration of glucose in the colon digesta of inoculated mice may also be due to slower transit in the SI, typical of GF mice [437], which may increase the efficiency of simple carbohydrate absorption.

4.8.3 Bacterial translocation

B. bifidum AGR2166 was not able to be cultured from maternal organs and foetuses by the end of the experimental period. Bifidobacteria administered orally to GF mice, however, have been reported to translocate to MLN, spleen, liver, lungs and kidneys kidneys only during the first three weeks after inoculation [438, 439]. The cessation of Bifidobacteria translocation was correlated to cell-mediated immunity [438]. During pregnancy the maternal immune system shifts from cell-mediated immunity [180] towards one characterised by humoral immunity [181]. This altered maternal immunological state may increase the translocation of bacteria and their components from the maternal GIT into the blood and lymphatic circulation [173]. Near term, thinning of the placental barrier increases nutrient, waste product and gas exchange efficiency between mother and foetus [182] and possibly contributes to the influx of commensal bacteria and their components present in the blood circulation. This influx may initiate the first adaptation of the foetal intestine for life outside the mother.

The presence of bifidobacteria DNA in the maternal plasma, liver, MLN and placenta but not in amniotic fluid or the foetus was demonstrated in this study. The lack of detection of the bacterial DNA in the spleen, amniotic fluid and foetus may be due to the low levels of bifidobacteria in these samples. Human and laboratory mammal foetuses swallow amniotic fluid, prior to the presence of milk in the GIT, therefore, bacteria in the amniotic fluid if any, are likely to be found in the foetal GIT. Rather than an inoculum for GIT colonisation, the low levels of bacteria previously reported in umbilical cord blood, amniotic fluid, placenta and foetal membranes are likely to stimulate the development of GIT mucosal immune system of the foetus [117], preparing for life outside the uterus.

The presence of bacterial DNA alone may stimulate the infant immune development. The presence of bacterial DNA alone may also stimulate the infant immune development. For example, CpG dinucleotides within bacterial DNA are known as potent immune stimulators, acting through toll-like receptor 9 [120]. Conversely, immune suppressive motifs including

poly-guanosine or guanosine cytosine-rich sequences, such as those on the telomere region of mammalian DNA, that can block immune activation induced by CpGs [165]. Recently, immune suppressive motifs (TTAGGG and TCAAGCTTGA) that are able to counter the effects of CpGs have been discovered in *Lactobacillus* [166]. If immune-modulatory motifs occur in the bacterial DNA translocated from the maternal GIT to the foetus, they could contribute to the immune development by decreasing exaggerated inflammatory responses to colonising bacteria, which are seen in infants with necrotising enterocolitis [167]. This exposure to the bacteria before delivery may educate the infant immune system through PRR CD14 and TLR [168] receptors that recognise bacterial cell wall components. These receptors may induce analogous responses to maternal antigens and protect the neonate against pathogens [169, 170].

Dams fed CMO diet had an increased bacterial translocation (bacterial DNA was detected in 8 samples of animals fed combo diet and 12 samples of animal fed CMO diet) compared to that of dams fed the combo diet. Dietary fibre has been reported to increase bacterial translocation [440, 441]. Dietary fibre and the bacterial end-products of fibre fermentation are important in maintaining the normal ecological balance of the GIT microbiota and have been shown to protect against bacterial translocation linked to infection [440, 441]. The dietary intake of CMO by the dams may have increased the *B. bifidum* population in the GIT and, consequently, increased its translocation to maternal organs, compared to that of the dams that received the combo diet.

4.9 Conclusions

To the best of our knowledge, this is the first study on the effects of an oligosaccharide enriched diet fed to mono-associated dams. Diet enriched with CMO, independent of bifidobacteria mono-association had no effects on dams GIT morphology and metabolism. The CMO diet also increased the translocation of bifidobacteria DNA from maternal GIT to organs and the placenta, indicating that CMO supplementation may stimulate the

translocation of beneficial bacteria from the maternal GIT to the foetal membranes to the benefit of the foetus. In agreement with the literature (which states that the bacterial colonisation of GF mice can revert some reproductive characteristics of GF mice to a state similar to conventional mice), the present study showed that GF mice inoculated with *B. bifidum* AGR2166 had increased foetus numbers and weight compared to non-inoculated mice. Caution is needed, however, when extrapolating results obtained from the study on the effects of dietary components on the GIT microbiota with mono-associated models. Further studies using conventionally raised mouse models are needed to develop a deeper understanding of the interactions between dietary CMO, the host, and bacteria.

Chapter 5 Effects of dietary caprine milk oligosaccharides on dam large intestine and the consequences for the development of the offspring

Chapter 5

5.1 Introduction

The GIT microbiota plays an important role in maintaining host health by preventing the colonisation of pathogens [385], fermenting dietary compounds [387], regulating lipid storage [389], and modulating mucosal immunity [384]. The composition of the microbiota profoundly influences the development and maturation of the infant mucosal immune system [442], and innate and adaptive immune responses (particularly in early life), and may affect health in later life [443, 444]. A higher risk of metabolic diseases in adults, for example, has been associated with changes in the GIT microbiota in early life [228, 399, 400]. Strategies to manipulate the microbiota during infancy may reduce the risks of developing some diseases as an adult.

The maternal microbiota is the main source of bacteria colonising the infant GIT during labour [169, 398] and breast feeding [246, 394, 445]. Emerging evidence suggests that these bacteria may also colonise the infant GIT before birth [114, 172, 395]. Phagocytes present in the maternal GIT can migrate from mucosa to the lactating mammary glands via lymphatic and blood circulation [169]. In early postnatal life, the type of feeding (breast vs formula), environment and any antibiotic treatment also play an important role in determining and maintaining the GIT microbiota composition [246, 394, 445].

Breast-fed infants receive a unique profile of complex natural HMO, known to: improve the colonisation of the GIT and immune responses [446]; stimulate health promoting bacteria [447]; prevent adhesion of pathogens to GIT epithelial cells [331]. Attempts to mimic the beneficial effects of HMO in the GIT have been made by enriching formula with synthetic oligosaccharides, such as FOS or GOS. Colonisation of the GIT by ‘less helpful’ microbiota, and susceptibility to GIT disease or sepsis are, however, still higher in formula-fed infants compared to breast-fed infants (reviewed in [448]).

Few studies to date have focused on manipulation of the maternal microbiota as a way of improving the infant's GIT function and reducing the risk of diseases when adult [228, 399, 400]. The manipulation of maternal microbiota (in humans and rodents), through antibiotics [31, 193], and diet [244, 246] have been shown to affect the bacteria transmitted to the offspring both quantitatively and qualitatively. Fujiwara et al. [27, 246], for example, demonstrated in mice that FOS supplemented to dams changed the GIT microbiota profile of the offspring, and diminished the severity of atopic dermatitis. In contrast, the combined dietary supplementation of GOS and FOS increased the proportions of bifidobacteria in the human GIT, but there was no effect on the infant [244]. Another study showed that a GOS and inulin enriched diet, fed throughout pregnancy and lactation, was able to increase colon length and thigh muscle mass in the offspring [247].

Natural prebiotic oligosaccharides, similar to HMO, have been found in the milk of all mammals studied so far [145, 301, 449]. Among domestic farm animals, caprine milk has a number of oligosaccharides structurally similar to human milk [265], with potentially similar beneficial effects on the human infant [450]. Dietary CMO were unable to stimulate the growth and fermentation of bifidobacteria mono-associated to dams in Chapter 4, however, the dietary effects of CMO on the complex microbiota from conventionally raised dams remains unknown.

5.2 Hypothesis and Aims

The first hypothesis of the research presented in this Chapter was that the consumption of CMO by the dams during gestation and lactation would promote a bifidobacteria enriched microbiota, lead to an improved bacterial fermentation in the caecum, and healthier morphology in the dam's large intestine. The second hypothesis was that these changes in the maternal large intestine would be accompanied by improvements in the dam's milk quality/quantity, and in the growth of the offspring and their GIT microbiota composition. The

third hypothesis was that the effects of maternal CMO supplementation on the offspring's growth and GIT microbiota composition are detected after 30 days consuming control diet.

The first aim was to determine the effects of a CMOP diet on the dam's large intestinal microbiota, SCFA production, plasma and urine metabolites, and milk composition. The second aim was to evaluate the effects of maternal diet on body weight, organ weight and length, femur mineral composition, plasma glucose and leptin concentration, and plasma and urine metabolites in pups at weaning. The third aim was to determine whether the effects of maternal diet on these parameters in pups at weaning persist following a change to feeding pups a control diet for 30 days.

5.3 Material and methods

5.3.1 Mice

63 C57BL/6 mice (42 female and 21 male) were obtained from the Ruakura Small Animal Facility at 6 weeks of age. A power analysis[1] showed that 5 C57BL/6J mice were required per group to obtain a detectable difference in caecal digesta SCFA concentrations (for 90% power and significance of 0.05), using a standard deviation of SCFA of 5 µmol/g, and Bifidobacteria and Lactobacillus counts of 0.2 (log CFU/g wet stool) [451, 452]. There are no previous studies with the information needed for a power analysis for changes in milk composition or plasma glucose concentration of the dam. This necessitated a cautious approach to estimating the number of animals required for the study. Because of the incomplete information available, and the uncertainty about similarity of conditions from those in the literature cited above, the number of animals per group was doubled. The number of mice matches those used in the published studies. Due to challenges in reaching

[1]Power analysis was calculated by John Koolaard, statistician at AgResearch, Palmerston North.

expected pregnancy rates, the number of mice was again increased by 30%. All mouse experiments were approved by the AgResearch Grasslands Animal Ethics Committee, Palmerston North, New Zealand (AE Approval 12579).

5.3.2 Housing

Mice were housed under conventional conditions (temperature of 22ºC, humidity 60%, air exchange 12 times/h, 12 h light/dark cycle) in PC2 containment. Food and drinking water were provided *ad libitum*. Cages, feeders, and water bottles were sterilised weekly and bedding changed as necessary. Refusals were weighed twice a week, and scattered refusals in the cage weighed when bedding was changed. Mice were monitored for signs of poor health (including weight loss, diarrhoea, blood in faeces, self-mutilation, skin irritation, coat condition, movement, and posture) using a General Health Score (GHS). Animals were scored daily, ranging from 1 (almost dead) to 5 (healthy/normal), once a GHS of 2 was attained; mice were immediately euthanised by carbon dioxide anaesthesia followed by cervical dislocation. Mice with a GHS of 3 were closely monitored, and if their condition did not improve, they were humanely euthanised. During the period between the last days of pregnancy and the first milk sampling, only food and water were supplied, and mice were not otherwise manipulated (to avoid stress, and high rates of cannibalism often observed).

5.3.3 Diet

All three diets used in this study were based on AIN-76A. The AIN-76A diet is a commonly used research diet, formulated to meet the requirements of adult mice and rats using bovine casein as the protein source [453]. AIN-76A (control diet), AIN-76A supplemented with GOS, lactose, glucose and galactose (combo diet) and AIN-76A supplemented with CMOP containing 2% CMO (CMO diet) were formulated by Research Diets, Inc. (New Jersey, USA

Table 5.1 Composition of caprine milk oligosaccharide enriched product (CMOP) added to the experimental diet.

Macronutrients (g/kg)		
Protein		10
Lipid		0.8
	GOS	110.7
	Lactose	201.5
Carbohydrates	Glucose	200.8
	Galactose	264.5
	Oligosaccharides	215
Micronutrients ((g/kg), except selenium (mg/kg))		
Calcium		0.1
Magnesium		0.1
Potassium		1.9
Sodium		0.9
Iodine		0.8
Selenium		<0.004

Combo contains sugars (GOS, lactose, glucose and galactose) at the same concentrations present in the CMOP (Table 4.2). The sucrose concentration was adjusted in the CMO and combo modified diets to balance the energy and nutritional content of the AIN-76A diet (Table 5.2). All ingredients of the AIN-76A, combo and CMO diet (except CMOP, sourced from New Zealand), and GOS (sourced from Yakult, Japan), were supplied by Research Diets. All diets were pelleted and colour coded, and stored at room temperature in a cool dry place until fed. CMOP are stable in room temperature.

5.3.4 Study design

At 6 weeks of age female mice were kept in large groups for two weeks (one week with no males nearby, and one week in the same room as the males), to bring all the females into oestrus at the same time (Figure 5.1). This procedure ensures that diestrus is prolonged, and oestrus is suppressed, a phenomenon known as the Whitten effect. When the females were placed with males, the oestrous cycle of most females will restart within three days, increasing the chance of the experimental group becoming pregnant on similar dates [454].

At 9 weeks of age mice were randomly assigned to groups of two females and one male, ensuring each group had a similar mean body mass index (BMI)[2] and standard deviation. 16 C57BL/6 female and eight male mice were fed control diet, 16 C57BL/6 female and eight male mice were fed combo diet and 10 C57BL/6 female and five male mice were fed CMO diet. The amount of CMOP available, and consequently, the amount of the CMO diet were limited, therefore 10 females and five males were used for the CMO treatment group (as many as could be fed for the experimental period). Mice were mated for 11 days (2 full oestrum cycles, usually four to five days per cycle). From day 12, the females were

[2] BMI were calculated as the body weight (g) / [nose-anus length (mm)].

Table 5.2. Diet composition

	AIN-76A	Combo	CMO
Diet composition (%)			
Protein	20	20	20
Carbohydrate	66	66	66
Fat	5	5	5
Diet composition (g/kg)			
Casein	200	200	200
Corn oil	50	50	50
Cellulose, BW200	50	50	50
DL-Methionine	3	3	3
Mineral Mix S10001	35	35	35
Vitamin Mix V10001	10	10	10
Choline Bitartrate	2	2	2
Corn Starch	150	150	150
Sucrose	500	427.7	407
Glucose (dextrose monohydrate)	0	18.7	18.7
Galactose	0	24.6	24.6
Lactose	0	18.7	18.7
Galacto-oligosaccharides	0	10.3	10.3
CMO-enriched fraction	0	0	20

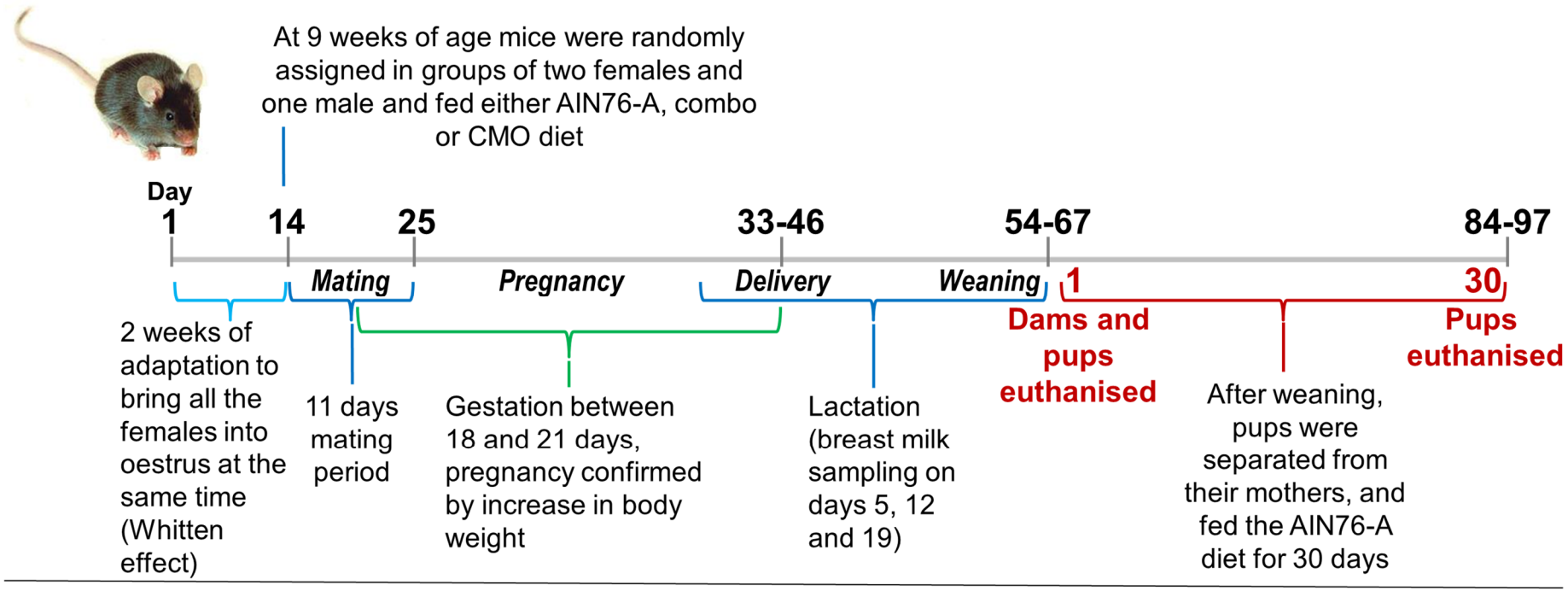

Figure 5.1 Study design

individually housed and the males humanely euthanised. Dams were weighed twice a week during the experimental period as part of the General Health Score and to confirm pregnancy. Weighing was used as a method to confirm pregnancy instead of other parameters generally used (such as the presence of a vaginal plug or a smear to detect the presence of sperm), to avoid animal stress. After two weeks, non-pregnant females were humanely euthanised. Dams were checked daily to determine delivery date.

After birth, all pups were milk fed by dams up to weaning (21 days after delivery). Samples of the dam milk were collected at lactation days 5, 12, and 19 to look for changes in milk composition. At weaning, pups from each dam were assigned to 2 groups containing equal numbers of females and males (Figure 5.2). All dams and half of the pups were humanely euthanised and sampled at weaning. To determine long term effects of maternal diet, the remaining pups were fed the control diet for 30 days, then euthanised and sampled (Figure 5.1).

5.4 Sampling

5.4.1 Milk samples

Pups were separated from their mothers one hour before milk sampling. Mice were lightly anaesthetised with 0.2 mL of ketamine/xylazine/acepromazine mix (50 mg/mL ketamine, 5 mg/mL xylazine and 0.5 mg/mL acepromazine), then injected with oxytocin (0.15 iu/mouse) subcutaneously to stimulate milk production and improve milk flow. Nipples were wiped with 70% ethanol before collection. Milking was done manually into a sterile tube, kept on ice and stored at -20°C until analysed. Milk samples obtained on each of the three sampling occasions (days 5, 12, and 19) were pooled prior to compositional analysis.

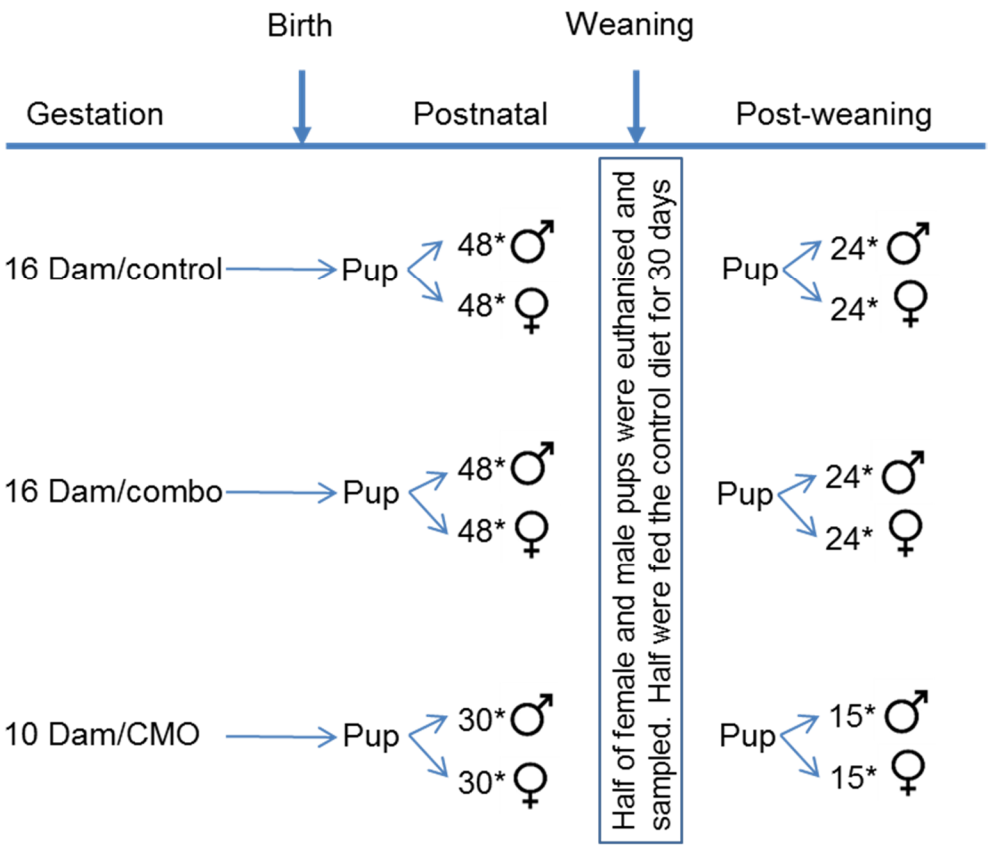

Figure 5.2 Theoretical distribution of the mice across the three experimental phases (gestation, postnatal and weaning). *Number of male and female pups based on the assumption that each female would have 6 pups (3 male and 3 female).

5.4.2 Urine samples

Urine samples for metabolomics analysis were collected from dams before mating and before euthanasia, and pups 30 days after weaning, before euthanasia. This procedure involves holding the mouse over a Petri plate and encouraging it to urinate by gentle trans-abdominal pressure over the bladder [455]. Each mouse may produce 30 to 100 µL of urine, which was immediately transferred to a sterile tube using a pipette, frozen in liquid nitrogen, and kept at -80°C for further processing.

5.4.3 Plasma and serum samples

Blood samples were collected from the dams from the tail tip with non-heparinised and heparinised capillary tubes before mating. The capillary tubes were sealed and centrifuged to produce serum (serum samples were left for clotting for 10 min at room temperature) and plasma samples. Plasma and serum samples were also prepared from dams and pups from blood collected by cardiac puncture after euthanasia. Plasma aliquots were collected in two different tubes, one kept on ice for glucose analysis, and one snap frozen in liquid nitrogen and kept at -80°C for metabolomics analysis. Serum samples were kept at -20ºC for leptin analysis.

5.4.4 Tissue samples

Mice were fasted for 14 h overnight, re-fed for 2 h and fasted again for 2 h before sampling to minimise variation in food intake and its affects on the parameters analysed [408]. Each mouse was sampled over approximately 15 min, so the fasting and re-feeding was staggered by one hour in groups of 4 to 5 mice.

Dams and offspring were euthanised by carbon dioxide anaesthesia followed by cervical dislocation. Blood was collected by cardiac puncture. The GIT was removed and the length and weight were recorded prior to full dissection. The stomach and caecum were cut from

the GIT and weighed. Caecum digesta was collected, snap frozen and kept at -80°C until SCFA analysis. The colon was removed and the length and weight were recorded prior to full dissection. Transverse sections of the proximal colon were collected and stored in 10% formalin solution at room temperature for subsequent assessment of the colonic crypt length and goblet cell number. Both femurs were removed from tissue using gauze, weighed and stored at -20°C for evaluation of bone mineral concentrations. Spleen, brain, kidneys and fat deposits (mesenteric, inguinal, epididymal and retroperitoneal) were removed and weighed.

5.5 Sample analysis

5.5.1 Diet composition

Diet composition, except individual sugars, were analysed by the Nutrition Laboratory Institute of Food, Nutrition and Human Health, Massey University (Palmerston North, New Zealand).

AOAC methods were used: dry matter and ash, AOAC 930.15/925.10/942.05 [306]; starch α-amylase, AOAC 996.11 [456]; total sugars, phenol and sulphuric acid [457]; protein, Leco, AOAC 968.06 (Dumas method, nitrogen - protein conversion factor = 6.25) [307]; fat, Mojonnier, AOAC 954.02 [308]; vitamin D2/D3, AOAC 2002-05 [458]; minerals, inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES); total, soluble and insoluble dietary fibre, Megazyme, AOAC 991.43 [459].

Oligosaccharide characterisation and quantification were analysed by LC-MS using a Thermo Scientific LTQ XL-Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionisation in negative mode (method described in Chapter 4). Lactose, glucose, galactose and GOS concentrations were analysed by high pressure ion chromatography (IC) using a DIONEX ICS-5000+ SP/DC (Thermo Fisher Scientific, Waltham, MA, USA) (method described in Chapter 4).

5.5.2 Histology

Sectioning and staining of histology slides was carried out by Diane Sebelin Histology Services, Mosgiel, New Zealand.

For dams, pups at weaning, and pups 30 days after weaning, three colon serial sections were analysed. Tissues were fixed in 10% phosphate buffered formaldehyde, dehydrated using a series of ethanol concentrations for 1 hour each (70%, 80%, 95%, 100%), incubated for 1 h in xylene, embedded in paraffin, sectioned to 5 µm thickness, and placed on a glass slide. Paraffin was removed from the sections with xylene, and the sections rehydrated with 100% and 95% ethanol and stained with haematoxylin for 3 to 5 min. Sections were then rinsed with 1% HCl in 70% ethanol, with 95% ethanol, and stained with eosin for 1 to 4 min. Finally, they were dehydrated twice in 95% and 100% ethanol and xylene, enclosed in mounting medium (Permount) and placed on slides. The slides were examined by light microscopy at 200 times magnification.

5.5.3 Caecal short chain fatty acids

Sample preparation was performed with the assistance of John Rounce and Bruce Sinclair, technical support from the Food Nutrition & Health Team, AgResearch, Palmerston North. GC-FID was run by Wade Mace, scientist from the Plant-Fungal Interactions team, AgResearch, Palmerston North.

Caecum digesta samples were thawed and approximately 100 mg of each sample was mixed with 1 mL of PBS and 30 µL of internal standard (200 mM/L 2-ethyl butyric acid). Samples were homogenised using a hand held homogeniser (OMNI international, Kennesaw, USA) at full speed for 10 s and then centrifuged (12000 g for 5 min at 4°C) to remove particulate material, following which 1 mL of supernatant was transferred to a glass tube. Extraction of SCFA into an organic solvent was performed by adding 0.5 mL hydrochloric acid and 2 mL diethyl ether followed by vortexing for 45 s. The samples were

then centrifuged (1000 x g for 10 min at 4°C) to separate the aqueous and organic phases. The upper diethyl ether phase was collected and samples derivatised by adding 40 µL N-methyl-N-E-butyldimethylsilytrifluoracetamide (MTBSTFA) (Sigma-Aldrich) and incubating at 80°C for 20 minutes. The samples were then left at room temperature for 48 hours prior to GC analysis to allow for complete derivatisation of lactic acid [460].

The concentrations of formic, acetic, propionic, butyric, isobutyric, lactic and succinic acids were analysed by flame ionisation detector and gas chromatography (GC-FID). The used of FID for quantification of fatty acid esters is advantageous in relation to other detector types because it is robust, possesses a wide linearity range, has a rapid response, and its limit of detection is 10^{-12}g (pg) for alkanes. A Shimadzu GC-2013 plus gas chromatograph system (Nakagyo-ku, Kyoto, Japan) fitted with a Restek Rtx-5Sil MS GC column (30 m × 0.25 mm × 0,25 µm) (Restek, USA) was used according to [460]). One µL of derivatised sample was injected at a 1:4 split ratio into the GC column with a helium carrier gas at 10 kPa for the first 6 min of the run, which increased to 15 kPa at 5 kPa per minute for the final 3 min. Detector and injector temperatures were both set to 260°C. The detector flame was maintained with a hydrogen/air mixture with both gases supplied at 50 psi. After an initial GC column temperature of 70°C at the time of sample injection, the temperature was increased to 80°C at 10°C per min, followed by an increase to 260°C at 20°C per min. After completion of the separation run, the GC column temperature was returned to 70°C. GC solutions software (Shimadzu) was used for data collection and chromatograph control. Identification and quantification of peaks were performed by running single standards of formic, acetic, propionic, butyric, lactic and succinic acids, and a standards cocktail containing all acids at a final concentration of 10, 5, 2.5, 1.25, 0.625, and 0.313 mM to generate a standard curves. All peak areas were normalised with a 5 mM 2-ethyl butyric acid internal standard, which was added to all samples and standards. Estimates of recovery of SCFA and carboxylic acids were performed by assaying samples spiked with known amounts of acids.

5.5.4 Plasma glucose concentration

Plasma glucose concentration was determined by colorimetry immediately after sample collection, using a glucose oxidase (GOD) based kit (LabAssay Glucose, Wako Japan). The hydrogen peroxide formed by GOD reacts with phenol and 4-aminophenazone in the presence of peroxidase (POD), to form a red - violet quinoneimine dye.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4H_2O$$

The assay was done in triplicate in a 96 well microplate using 2 µL of plasma per well. A standard curve was prepared by diluting standards to a final concentration ranging from 50 to 500 mg/dL. 300 µL of the chromogenic reagent was added to the sample or standard, and the plate mixed and incubated at 37°C for 5 min. Absorbance was measured at 505 nm. The limit of sensitivity of this assay is 50 mg/dL, and specificity is less than ± 12% when measuring the known concentration of a control serum.

5.5.5 Serum leptin concentration

Plasma leptin concentrations were measured using a Mouse Leptin Elisa kit (Millipore, Thermofisher NZ) [79] according to the manufacturer's instructions. This assay is a sequential Sandwich ELISA based on: 1) binding of leptin in the sample by a pre-titered antiserum and immobilisation of the resulting complexes in the wells of a microtiter plate; 2) after washing, biotinylated detection antibody binding to the immobilised leptin; 3) binding of horseradish peroxidase to the immobilised biotinylated antibodies after free detection antibodies are washed off; 4) washing off free enzyme conjugates, and 5) quantification of immobilised antibody enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity was measured spectrophotometrically by the increased absorbance at 450 nm, corrected from

the absorbance at 590 nm, after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured leptin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay using reference mouse leptin standards of known concentrations (0.2, 0.5, 1, 2, 5, 10, 20 and 30 ng/mL). Assays were done in duplicate in a 96 well microplate, using 10 µL of plasma per well. The limit of sensitivity of this assay is 0.05 ng/mL (~3.13 pM) leptin (10 µL sample size).

5.5.6 Milk nutrient composition

5.5.6.1 Dry matter

The dry matter was measured in triplicate on 10 µL of milk in pre-combusted and pre-weighed tin capsules. After weighing, the samples were dried at 55°C for 5 h, cooled to room temperature, and reweighed, and the dry matter calculated.

5.5.6.2 Sugar

Aliquots of 20 µL of milk were diluted 5 times with Milli-Q water and centrifuged at 21,200 x g for 20 min at 4°C to separate fat and whey. 60 µL of whey was transferred to a clean 1.5 mL tube, diluted 10 times with Milli-Q water and centrifuged at 16,100 x g for 10 min at 4°C. 50 µL of diluted whey was separated isocratically by HPLC using a Shimadzu LC10A HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand) fitted with a Bio-Rad Aminex HPX 87H HPLC column (maintained at 45°C) and a Shimadzu refractive index detector, RID10A. The mobile phase was sulphuric acid (5 mM) and an isocratic elution with a flow rate of 0.8 mL/min was used, with a run time of 15 min. Lactose, glucose, and galactose concentration were quantified based on concentrations of external standards at a final concentration from 0.01 to 0.05 mg/mL glucose and galactose; 0.2 to 1 mg/mL lactose.

5.5.6.3 Protein

The total protein concentration of milk was determined using a Bradford assay [461]. α-casein (Sigma, Aldrich, Auckland; 70% a casein, 30% other proteins) was used to produce the standard curve ranging from 0.05 to 0.2 mg/mL. 5 µL of milk was diluted 1000 times with Milli-Q water. 1 mL of Coomassie Blue reagent was added to the milk samples and standards, mixed and incubated for 5 min. The Bradford assay, a colorimetric protein assay, is based on the absorbance shift of the dye Coomassie Brilliant Blue, which under acidic conditions goes from the red form to its bluer form and binds to the protein being assayed. The binding of the protein stabilises the blue form, and the amount of the blue complex (directly related to the protein concentration) can be measured at 595 nm. All milk samples were measured in triplicate.

5.5.6.4 Fat

100 µL of milk was transferred to a 20 mL glass tube and diluted 10 times with Milli-Q water. 200 µL NH_3 solution (25%), 1 mL ethanol (100%), 3 mL diethyl ether, 3 mL petroleum ether (40 to 60°C), and 800 µL Milli-Q water were added, with 30 s vortexing after each step. The sample was then centrifuged at 3000 g for 10 min. 4 mL of the upper layer was transferred to a pre-weighed and pre-combusted glass vial and dried by nitrogen evaporation. The samples were dried for 2 h at 105°C, cooled in a dissecator, and weighed to determine the fat percentage**.**

5.5.7 Femur mineral composition

Femur digestion and sample preparation were performed by John Rounce, technical support from Food Nutrition & Health Team, AgResearch, Palmerston North. Hill Laboratories, Hamilton analysed the samples.

Both femurs were cleaned of all adherent tissues, weighed, then defatted and dried using two 16 h exposures to acetone and then ethyl ether. These were air dried and any obvious remaining cartilage material was removed [462]. Samples were placed in acid-leached, pre-weighed 20 mL borosilicate scintillation vials with acid-leached polypropylene caps and frozen at -80°C for 1 h. They were freeze dried overnight (FTS Systems, SP Scientific, Philadelphia, USA) and the dry weight recorded.

All samples received 2.5 mL HNO_3 (67% Aristar BDH #450043X) and 0.5 mL HCl (34 to 37%, Trace Metal Grade Fisher #FSBA508-P212) and heated in a foil covered aluminium block with acid leached funnels at 85°C for 1 h. Funnels were removed, and samples were heated up to 110°C and taken to dryness. 5 mL of analytical matrix (i.e. 1 mL HNO_3 (67% Aristar BDH #450043X): 1 mL HCl (34-37% Trace Metal Grade Fisher #FSBA508-P212): 5 mL H_2O) was added and weighed, and samples decanted to acid-leached polypropylene 10mL tubes for analysis.

Blanks were used to control for background levels in all steps (de-fatting, lyophilisation and digestion). Samples were digested alongside blanks and Certified Reference Material (CRM) i.e. IAEA-H-5 (Animal bone), to test for contamination and to calculate recovery rates (Ca, 96%-98%; Mg 93-100% and Zn 100%). Samples were measured on Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Electron Corporation, England) for calcium, magnesium and zinc concentrations (limit of detections: Ca 1.0 g/m^3, Mg 0.4 g/m^3, Zn 0.02 g/m^3).

ICP-MS was used instead of ICP-OES to compensate for the Easily Ionisable Elemental Spectral Effects on the ICP-OES. Calcium, for example, would have influenced the other elemental measurements, which would have necessitated matrix matched calibration standards [301].

5.5.8 Urine and plasma metabolomes

LCMS was run with assistance of Linda Samuelson, scientist from the Food Nutrition and Health Team, AgResearch, Palmerston North.

Nutritional metabolomics is a rapidly growing field using chemical profiling to look at metabolite effects of diet and nutrition in complex bio-systems [402]. Metabolite analysis can be classified as targeted or untargeted. Targeted analysis is a robust, reproducible, quantitative and accurate approach which analyses a small well defined list of compounds. The results of this analysis are, however, heavily biased towards the target metabolites, and probably reducing the rate of discovery of new, possibly important metabolites in biological systems. An untargeted metabolomics approach, on the other hand, is an unbiased method, favouring scope broadness and better understanding of the real effects of a nutritional intervention [463].

The analytical techniques developed for metabolomics usually include efficient chromatographic separations (e.g. capillary electrophoresis, gas chromatography, HPLC) and a powerful detection method (e.g. mass spectrometry (MS), nuclear magnetic resonance (NMR)). NMR is the only non destructive detection technique, and does not rely on separation of the analytes, and the sample can be recovered for further analyses. The main advantages of NMR are high analytical reproducibility and simplicity of sample preparation [464], although It is relatively insensitive compared to mass spectrometry based techniques. Mass spectrometry currently provides the most sensitive method to detect the broad range of chemicals. MS detects chemicals as ions in the gas phase, and this presents practical limitations to absolute quantification of a spectrum of chemicals. Molecules differ considerably in the conditions required for ionization in the gas phase. The energy required to create ions can result in reactions that convert metabolites to different chemical species. Furthermore, a single chemical can form multiple ionic forms, and relative amounts of these forms can vary due to the presence of other chemicals. These limitations are controlled by

using standardized separation techniques (gas chromatography, liquid chromatography, capillary electrophoresis), which simplify the mixture of chemicals introduced into the mass spectrometer at any time, and by careful choice of ionization methods [465].

Untargeted analysis of the urine and plasma metabolites was carried out using LC-MS. Urine (10 µL) was diluted with 200 µL of the internal standard solution (0.01 mg/mL d_2-tyrosine and 0.001 mg/mL dichlorofluorescein) into a glass vial before injection into the LC-MS. Plasma samples (100 µL) were mixed to 300 µL acetonitrile/methanol/acetone solution and kept on ice for 20 min. Samples were centrifuged at 14000 g for 10 min at 4°C and 250 µL transferred into a glass vial. Solvent was evaporated using a centrifugal evaporator (Savant SpeedVac, Thermo Scientific) and 100 µL of the internal standard solution (0.01 mg/mL d_2-tyrosine and 0.001 mg/mL dichlorofluorescein) added to each of the glass vials. Randomised batches of samples for each animal per treatment were analysed sequentially.

The Thermo LC-MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisted of an Accela 1250 quaternary UHPLC pump, a PAL auto-sampler fitted with a 15000 psi injection valve (CTC Analytics AG., Zwingen, Switzerland) and 20 µL injection loop, and an Exactive Orbitrap mass spectrometer with electrospray ionisation. A Merck polymeric bead-based ZIC-pHILIC column (HILIC) (100 mm, 2.1 mm, 5 µm) was used for the chromatographic separation of polar and hydrophilic compounds. The samples were separated at 25°C with a gradient elution program and a flow rate of 250 µL/min. The mobile phase was a mixture of acetonitrile-formic acid (99.9:0.1, v/v) (solvent A) and water–ammonium formate (16 mM, pH 6.3) (solvent B). The gradient elution programme was: held at 97% A (0 to 1 min), 97 to 70% A (1 to 12 min), 70 to 10% A (12 to 14.5 min), held at 10% A (14.5 to 17 min), returned to 97% A (17 to 18.5 min) and allowed to equilibrate for a further 5.5 min prior to the next injection. The samples were cooled in the auto-sampler at 4°C and the injection volume of each sample was 2 µL. The first 1.5 min and the last 5 min of the chromatogram were diverted to waste.

Data was collected in profile data acquisition mode over a mass range of m/z 55 to1100 at a mass resolution setting of 25000 with a maximum trap fill time of 100 ms using the Xcalibur software package provided by the manufacturer. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were as follows: spray voltage, 3.5 kV; capillary temperature, 325°C; capillary voltage, 90 V, tube lens 120 V. Negative ion mode parameters were as follows: spray voltage, -3.0 kV; capillary temperature, 325°C; capillary voltage, -90 V, tube lens -100 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5 [462].

To separate hydrophobic compounds, reverse phase LC-MS (RP LC-MS) analysis was performed using an Agilent RRHD SB-C18 column (C18) (150 mm × 2.1 mm, 1.8 µm) at 25°C with a gradient elution programme and a flow rate of 400 µL/min. The mobile phase was a mixture of water–formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile–formic acid (99.9:0.1, v/v) (solvent B). The gradient elution programme was as follows: held at 5% B (0 to 0.5 min), 5 to 99% B (0.5 to13 min), held at 99% B (13 to15 min), returned to 5% B (15 to 16 min) and allowed to equilibrate for a further 4 min prior to the next injection. The first 1.5 min and the last 6 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a mass range of m/z 60 to1200, at a mass resolution setting of 25000 with a maximum trap fill time of 100 ms using the Xcalibur software package provided by the manufacturer. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were as follows: spray voltage, 3.5 kV; capillary temperature, 325°C; capillary voltage, 50 V, tube lens 120 V. Negative ion mode parameters were as follows: spray voltage, − 3.5 kV; capillary temperature, 325°C; capillary voltage, −90 V, tube lens −80 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5 [462].

Components eluting between 3 and 14 min for the RP LC-MS analysis and 3 and 18 min for the hydrophilic interaction liquid chromatography (HILIC) analysis were extracted and aligned from the LC-MS data using PhenoAnalyzer (SpectralWorks Ltd, Manchester, UK). Key peak detection settings for the RP streams were: area threshold of 100000; peak width threshold of 0.06 min minimum width to 0.4 min maximum width; m/z peak detection window of 10 ppm. Key peak detection settings for the HILIC streams were: area threshold of 100000; peak width threshold of 0.1 min minimum width to 1.2 min maximum width; m/z peak detection window of 10 ppm. The resulting peak area matrix data files were de-isotoped by fitting the following criteria for the detection and removal of probable singly charged isotope ions.

5.5.9 Colonic microbiota

Sequencing was performed by Macrogen inc, Seoul, Republic Of Korea.

Analysis of the microbiota composition in colonic digesta was carried out using next generation sequencing (NGS). The major advantage offered by NGS technologies over traditional capillary sequencing platforms is the generation of much greater numbers of sequences without cloning bias by parallelising the sequencing process, enabling the production of thousands or millions of sequences at once. Three NGS platforms are widely used: the Roche 454 platform (Roche Life Sciences), the Applied Biosystems SOLiD platform (Applied Biosystems), and the Illumina (formerly known as Solexa) Genome Analyser and HiSeq platforms (Illumina). For these three NGS platforms, template DNA is fragmented, bound to adaptors, amplified by polymerase chain reaction, and subsequently immobilised on beads or on an array where clusters consisting of identical DNA fragments are formed. These clusters are read by sequential cycles of nucleotide incorporation, washing, and detection, where the number of cycles eventually determines the read length (Figure 5.3) [466]. Analysis of the large datasets generated by NGS platforms presents unique challenges. It is computationally intensive and the error rate is higher than with

traditional Sanger sequencing, which have been reported between 0.001% and 1% , while 454 sequencing has had reported error rates between 0.49% and 2.8% [316]. Due to the large number of sequences generated, an increase in error rate can result in a major overestimation of community diversity [305]. Therefore, stringent filtering of sequences based on quality score or complex algorithms for minimising the effects of sequencing errors is often required [454]. Despite these limitations, NGS platforms such as Roche 454 Genome Sequencer FLX Titanium, utilised in this study, have been proven to be invaluable for the compositional and metagenomics analysis of the GIT microbiota [321, 467].

The multiplexing of pyrosequencing by DNA-bar-coding techniques enabled the simultaneous characterisation of the colonic microbiota of mice fed control, combo and CMO diets. Firstly, approximately 50 mg of colon digesta DNA was extracted using a NucleoSpin Soil kit (Macherey Nagel). The amplification of a variable region of the 16S rRNA gene, V456, was done using a fixed primer at one end HTSt5 (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3') and a second primer carrying a unique key of 8 base pairs to assign the sequence reads to a given sample (HTSt103 to 190 primers). Amplification products (604 bp) were then evaluated by running on a 2% agarose gel.

The variable region of the 16S rRNA gene, V456, from the extracted DNA was amplified by PCR with each reaction mixture containing 1 µL of pooled DNA template (10 mM final concentration), Tris-HCl (2.5 mM final concentration), $MgCl_2$ (50 mM final concentration) KCl (each deoxynucleoside triphosphate at a final concentration of 200 mM), 20 pmol of primers HDA1 and HDA2, and 2.5 U of Taq DNA polymerase (Roche) in a total volume of 50 µL. The following program was used in a Hybaid Express thermal cycler (Thermo Fisher Scientific)

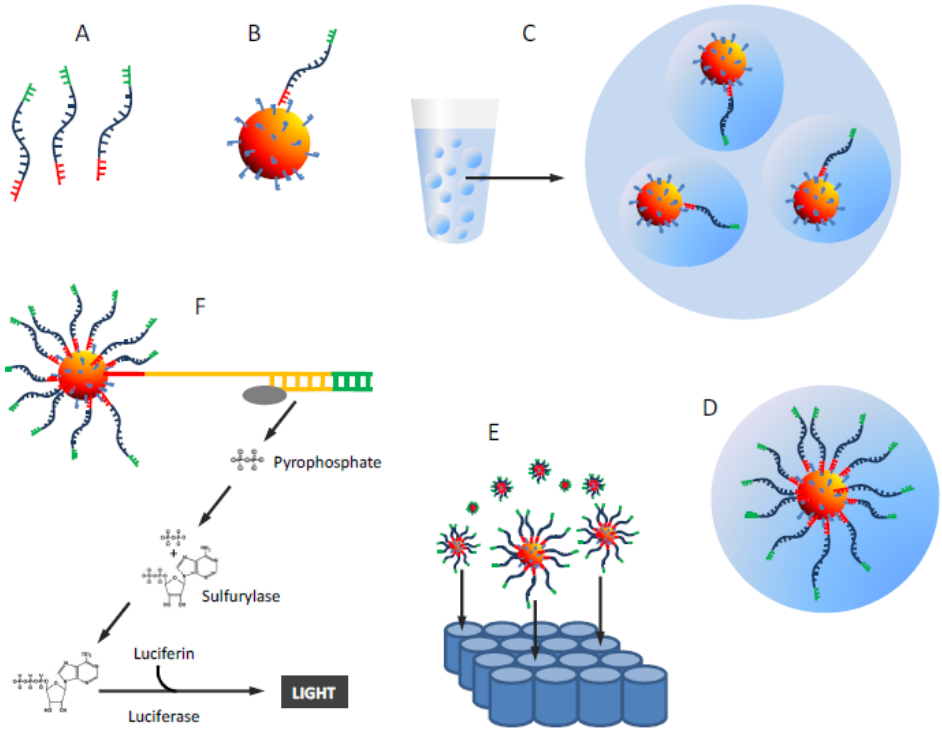

Figure 5.3 Next generation sequencing, Roche 454 workflow. (A) DNA samples are ligated with A and B adapters specific to the 5' and 3' ends. (B) Ligated DNA fragments are captured on microbeads. Concentrations of microbeads and DNA are optimised such that one microbead will have one captured DNA fragment. (C) Microbeads are suspended in a water-in-oil emulsion with amplification reagents, such that each microbead is contained within its own mini-reactor. (D) Each captured DNA fragment is amplified by emulsion PCR. (E) Microbeads with clonally amplified DNA are loaded onto a sequencing plate with a well diameter that allows only one microbead to fit in each well. Individual nucleotides are flowed across the plate in a fixed order to facilitate sequencing by synthesis. (F) Within each well, the introduction of a nucleotide complementary to the microbead bound DNA template results on the extension of a DNA strand by DNA polymerase. This results in the release of pyrophosphate, which is detected as a light signal following a chain of chemical reactions involving sulfurylase and luciferase. Diagram and workflow obtained from Young, 2011, [468].

94°C for 3 min, 30 cycles consisting of 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s, followed by 68°C for 7 min. The PCR products were then cleaned using a QIAquick PCR purification kit (Qiagen) using the following protocol. PB Buffer at a volume of 225 µL was added to each PCR reaction tube and mixed by vortexing. The samples were transferred to QIAquick PCR purification kit spin columns on 1.5 mL centrifuge tubes. The spin columns were then centrifuged at 5600 x g for 1 min. The flow through was discarded and the sample was then washed by adding 750 µL of PE Buffer to the column and centrifuging at 5600 x g for 1 min. The spin columns were placed in new microcentrifuge tubes and the DNA eluted by adding 30 µL buffer EB to each column. Samples were left to stand for 1 min after which they were centrifuged at 5600 x g for 1 min. Amplicons from 120 different biological samples were sent for Roche 454 sequencing in two pools (Pool 1, 58 samples, Pool 2, 62 samples) and the individual sample data sorted by the unique barcodes. Samples were separated in 2 pools (Pool 1 and Pool 2) because the number of barcodes available was less than the sum of all samples.

5.5.9.1 Analysis of 454 sequence data

Sequencing data analysis was performed with assistance of Wayne Young, scientist from the Food Nutrition & Health Team, AgResearch, Palmerston North.

The Pool 1 and Pool 2 454 datasets (16S rRNA gene amplicons) were sequenced using the GS FLX platform with Titanium chemistry. Amplicons were sequenced on two half regions and the resulting reads were processed using QIIME (version 1.8) pipeline (Quantitative Insights Into Microbial Ecology, [468]). Sequences were demultiplexed and quality filtered and chimera removal using default settings. The cleaned sample files were merged into a single Fasta file (keeping the sample information in each read entry) and subjected to downstream processing. Reads were then chimera checked using the USEARCH method against the Greengenes database (version GG_13_8). Sequences identified as chimeric

were removed from further analyses. Chimeras are hybrid products between multiple parent sequences that can be falsely interpreted as novel organisms, thus inflating apparent diversity. Sequences were clustered into operational taxonomic units (OTUs) using the UCLUST method at 0.97 similarity. Representative sequences were aligned using the PyNAST algorithm and assigned a taxonomy using the RDP classifier with a 0.8 confidence cut-off.

5.6 Statistical analysis

All statistical analysis except for the microbiota and metabolomics data were performed by testing the samples for normality and homogeneity of variances using ANOVA in GenStat version 12 (VSN International Ltd). Data were normally distributed and had homogeneous variances. Maximum 5% LSD was used to determine whether differences between groups were significant ($P < 0.05$). Trends were defined as $P > 0.05$ but < 0.1.

The metabolomics data were analysed using the online data analysis package MetaboAnalyst 2.5 (Xia, Mandal, Sinelnikov, Broadhurst, & Wishart, 2012), a web-based analytical pipeline for high-throughput metabolomics studies. Missing values were replaced with half the minimum value of each respective mass feature, auto-scaled (mean centred and divided by the standard deviation of each analyte) and a filter applied to remove 40% of the least varying features based on an interquartile range (25% for HILIC -ve as there were less analytes detected). For validation of significant components, peak areas were re-extracted from the original Thermo data files using Xcalibur with a 5 ppm window for further statistical analysis using MetaboAnalyst (version 2.5, online software) and R where required. The analysis of the metabolomics data were performed using ANOVA and Fisher's least significant difference method (Fisher's LSD). Differences between features were considered significant when probability was less than 0.005 ($P < 0.005$). The significance of the top 15 significant features of the metabolite markers based on the VIP (Variable Importance in Projection) scores of PLS-DA were also evaluated.

The analysis of the colon microbiota sequencing data was performed using ANOVA and Kruskal-Wallis test in R (V.3.0.2). Differences between means were considered significant when probability was less than 0.05 ($P < 0.05$).

Samples from mice fed AIN-76Adiet were identified using the control. Samples from mice fed combo and CMOP containing diet were identified using the term combo and CMO respectively.

5.7 Results

5.7.1 Changes in diet composition

Formulated diets were analysed in order to confirm that the macronutrient composition was as calculated for the test components (Table 5.3). The levels of protein (control, 16.2%, combo, 17.8%; CMO 18% ± 1.5%) and starch (control, 12.4%, combo, 11.8%; CMO 12.1% ± 0.4%) were lower than expected (protein, 20%; starch, 15%) in all diets. However, the levels of fat (control, 7%, Combo, 6.2%; CMO 5.9% ± 2.2%) were higher than anticipated for all diets (5%). CMO diet had higher levels of soluble (control, 0.4%, combo, 0.4%; CMO 0.6%), insoluble (control, 5.3%, combo, 5.4%; CMO 5.6%) dietary fibre compared to control and combo diet. CMO were present at only 1% instead of the 2% expected for this diet.

Lactose, glucose and galactose were present in lower concentrations than expected in the combo and CMO diet. The combo diet, however, had double the amount of glucose and galactose, twenty four times more lactose and five times more GOS than the CMO diet. The control diet also contained lactose (0.08 g/kg), glucose (1.2 g/kg) and galactose (5.5 g/kg).

Mineral and vitamin D2 and D3 composition was also evaluated. Vitamin D3, was originally added to the diet at concentration of 1 IU/g, however, this concentration falls outside the limited of detection (1.2 IU/g) for the analytical method applied. Vitamin D2 is not described in the list of diet components but the analysis revealed the presence of this vitamin in all

Table 5.3 Nutrient composition of diets as tested at the end of the experiment. Expected values from the diet formulation are shown in brackets.

	AIN76-A	Combo diet	CMO diet
Diet composition	**%**	**%**	**%**
Dry matter	96.3	95.7	94.9
Ash	2.8	2.8	3.3
Protein	16.2 (20)	17.8 (20)	18.0 (20)
Starch	12.4 (15)	11.8 (15)	12.1 (15)
Fat	7.0 (5)	6.2 (5)	5.9 (5)
Soluble dietary fibre	0.4	0.4	0.6
Insoluble dietary fibre	5.3	5.4	5.6
Total dietary fibre	5.7	5.7	6.2
Total Sugars (g/100g)	47	47	45
Test components	**g/kg**	**g/kg**	**g/kg**
GOS	0 (0)	10.6 (10.3)	2 (10.3)
Lactose	0.08 (0)	14.2 (18.7)	0.6 (18.7)
Glucose	1.2 (0)	13.8 (18.7)	7.9 (18.7)
Galactose	5.5 (0)	15.9 (24.6)	8.6 (24.6)
Caprine milk oligosaccharide	0 (0)	0 (0)	11 (20)
Vitamin	**IU/g**	**IU/g**	**IU/g**
Vitamin D2 (IU/g)	3.0 (0)	4.0 (0)	4.2 (0)
Vitamin D3 (IU/g)	<1.2 (1)	<1.2 (1)	<1.2 (1)
Mineral			
Calcium (g/kg)	5.2 (5.2)	5.4 (5.2)	5.4 (5.2)
Magnesium (g/kg)	0.52 (0.5)	0.53 (0.5)	0.60 (0.5)
Zing (mg/kg)	45 (29)	42 (29)	41 (29)

three diets (control, 3 IU/g, combo, 4.0 IU/g; CMO 4.2 IU/g ± 12.6%). Calcium and magnesium levels were close to the anticipated level for all diets. However, zinc concentrations were higher (control, 45 mg/kg, combo, 42 mg/kg; CMO 41 mg/kg) than expected (29 mg/kg) in all diets.

5.7.2 Dams and litters

Of the 10 mice originally assigned to the CMO treatment and 16 assigned to combo and control treatments, 8 females on CMO, 14 on combo and 11 on control were pregnant by the end of the mating period. During pregnancy, weight gain was monitored up to 2 or 3 days before delivery to avoid dam stress; therefore no comparisons on weight gain during pregnancy were able to be drawn. After delivery, the dams were weighed before milk sampling (at day 5, 12 and 19). No significant difference in dam weight was found among the treatment groups (Table 5.4). Food intake was also similar for all groups (control, 3.87; combo, 4.03; CMO, 4.22 ± 0.21; mean [g/d] ± SE)

After delivery, cannibalism occurred in 3, 4 and 1 dams fed control, combo and CMO diet, respectively. These dams were euthanised and excluded from all analysis. The number of pups born in each treatment was only counted 5 days after birth, to avoid causing additional stress to the nursing dams. Therefore, it is not known if any cannibalism occurred during this period and no comparisons could be drawn. The number of nursing dams and pups at weaning and 30 days after weaning are described in Figure 5.4. No difference was found among the number of remaining pups alive at weaning (control, 5.7; combo, 4.0; CMO, 5.4 ± 0.6; mean ± SE). Intake of control diet by pups from weaning up to 30 days post-weaning were measured and no differences were found among pups from dams fed control, combo and CMO diet (control, 46.07; combo, 49.32; CMO, 49.96 ± 2.5; mean [g/d] ± SE, P = 0.2).

Table 5.4 Average dams body weight at day 5, 12 and 19th after delivery among the treatments.

	Control	Combo	CMO	SE	P-value	LSD
Day 5	23.3	22.8	23.8	0.7	0.48	1.5
Day 12	25.2	24.7	24.6	0.8	0.71	1.6
Day 19	25.5	25.7	25.6	0.7	0.90	1.5

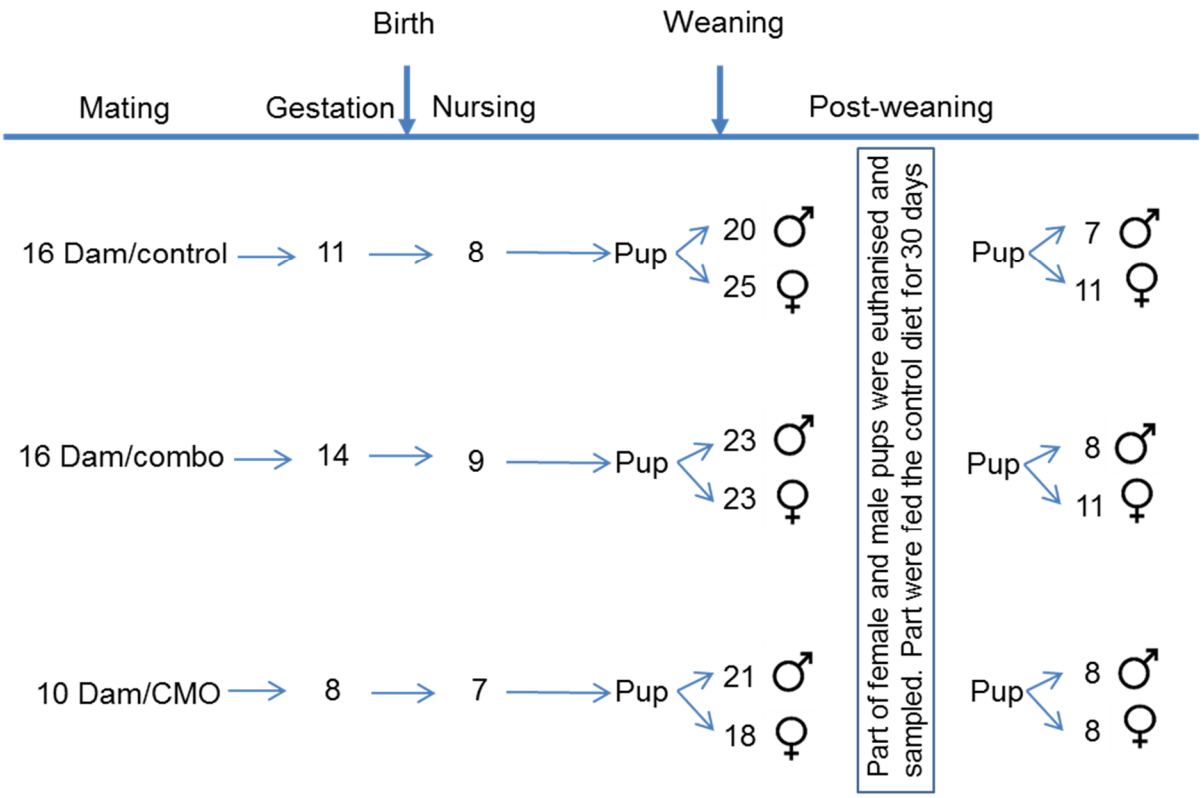

Figure 5.4 Number of dams across the three experimental phases (mating, gestation and nursing) and distribution of pups at weaning and 30 days after weaning.

5.7.3 Body and organ weight and length

It was expected that there would be a significant sex effect for body and organ weight in animals at weaning and 30 days after weaning (i.e. males higher than females), thus, all organ weights and lengths were expressed as proportion of individual body weight, which normalises each animal to their respective total body weight. There was no evidence that diet affected dams GIT length, SI length and stomach, spleen, kidneys, brain, femur and visceral fat weight and normalised weight (Table 5.5) during the experimental period (from mating to weaning). However, dams fed the CMO diet had a higher absolute colon length (control, 34.9; combo, 41.3; CMO, 47.0 ± 4.0; mean [cm] ± SE; P = 0.02) and normalised colon length (control, 1.4; combo, 1.7; CMO, 1.9 ± 0.2; mean [cm/g] ± SE; P = 0.05) compared to the dams fed the control diet. Feeding the CMO diet decreased absolute liver weight (control, 2137; combo, 2177; CMO, 1779 ± 149; mean [mg] ± SE; P = 0.03) and normalised weight (control, 85.7; combo, 85.9; CMO, 73.0 ± 5.4; mean [mg/g] ± SE; P = 0.05) of the dams. At weaning, pups from dams fed CMO and combo diets, however, had an increased absolute liver weight (control, 157.6; combo, 178.7; CMO, 187.5 ± 149.0; mean [mg] ± SE; P = 0.03) compared to pups from dams fed the control diet.

Although, dam body weight and length were not affected by diet during the experimental period (from mating to weaning), pups at weaning, from dams fed the CMO diet had increased body weight (control, 4.7; combo, 5.0; CMO, 5.2 ± 0.2; mean [g] ± SE; P = 0.02) compared to the pups from dams fed the control diet (Table 5.6). Body length was increased in weaned pups from dams fed the CMO and combo diets (control, 5.2; combo, 5.4; CMO, 5.4 ± 0.1; mean [cm] ± SE; P = 0.04) compared to pups from dams fed the control diet. Pups weaned from dams fed CMO had increased GIT length (control, 214.0; combo, 217.8; CMO, 227.5 ± 4.1; mean [cm] ± SE; P = 0.006) as well as absolute (control, 14.2; combo, 16.1; CMO, 23.9 ± 1.6; mean [cm] ± SE; P < 0.001) and normalised colon length (control, 3.1; combo, 3.3; CMO, 4.5 ± 0.7; mean [mm/g] ± SE; P < 0.001) compared to pups from dams

Table 5.5 Absolute and normalised mean body and organ weight and length in dams.

	Control	Combo	CMO	SE	P value	LSD
Body weight (g)	24.9	25.3	24.5	0.8	0.6	1.8
Body length (cm)	8.9	8.8	8.8	0.1	0.7	0.3
Absolute organs length and weight						
GIT length (mm)	439.8	441.4	422.0	13.9	0.3	28.8
SI length (mm)	368.1	366.8	351.7	13.5	0.4	28.1
Colon length plus rectum (mm)	71.6	75.2	70.9	3.0	0.2	6.2
Colon length (mm)	34.9[a]	41.3[ab]	47.0[b]	4.0	0.02	8.4
Stomach weight (mg)	1548.0	1143.0	1203.0	351.7	0.4	731.3
Colon weight with contents (mg)	306.1	310.7	323.8	29.0	0.8	60.4
Colon weight no contents (mg)	159.3	180.1	169.4	13.6	0.3	28.4
Liver weight (mg)	2137.0[a]	2177.0[a]	1779.0[b]	149.0	0.03	309.9
Spleen weight (mg)	62.4	66.4	63.9	6.6	0.8	13.7
Kidneys weight (mg)	336.5	328.0	325.3	14.2	0.7	29.5
Brain weight (mg)	429.9	433.3	430.4	10.0	0.8	20.8
Femur weight (mg)	141.2	151.7	151.9	7.5	0.2	15.7
Visceral fat weight (mg)	2287.0	2370.0	2318.0	364.8	0.9	550.7
Relative organs length and weight per mouse weight						
GIT length (mm g^{-1})	17.7	17.5	17.3	0.7	0.8	1.4
SI length (mm g^{-1})	14.8	14.5	14.4	0.6	0.7	1.3

Colon length plus rectum (mm g^{-1})	2.9	3.0	2.9	0.2	0.7	0.3
Colon length (mm g^{-1})	1.4^{a}	1.7^{ab}	1.9^{b}	0.2	0.05	0.4
Stomach (mg g^{-1})	61.4	46.6	46.9	13.2	0.4	27.4
Colon weight with contents (mm g^{-1})	12.3	12.3	13.3	1.2	0.6	2.5
Colon weight no content (mg g^{-1})	6.4	7.1	7.0	0.6	0.4	1.2
Liver (mg g^{-1})	85.8^{a}	86.0^{a}	73.1^{b}	5.4	0.05	11.3
Spleen (mg g^{-1})	2.5	2.6	2.6	0.3	0.9	0.6
Kidneys (mg g^{-1})	13.5	13.0	13.3	0.4	0.4	0.9
Brain (mg g^{-1})	17.2	17.2	17.6	0.6	0.6	1.2
Femur (mg g^{-1})	5.7	6.0	6.2	0.3	0.2	0.6
Visceral fat weight (mg g^{-1})	92.0	93.2	94.6	9.2	0.9	19.2

Dams (n= Control, 8; Combo, 9; CMO, 7). Values with similar letters in rows do not differ significantly ($P < 0.05$).

Table 5.6 Absolute and normalised mean body and organ weight and length in weaned pups from control, combo and caprine milk oligosaccharides-fed dams.

	Control	Combo	CMO	SE	P value	LSD
Body weight (g)	4.7[a]	5.0[ab]	5.2[b]	0.2	0.02	0.4
Body length (cm)	5.2[a]	5.4[b]	5.4[b]	0.1	0.04	0.2
Absolute organs length and weight						
GIT length (mm)	214.0[a]	217.8 [a]	227.5 [b]	4.1	0.006	8.3
SI length (mm)	177.5	177.8	181.1	3.6	0.5	7.2
Colon length plus rectum (mm)	36.8	36.9	36.6	1.3	0.07	2.6
Colon length (mm)	14.2 [a]	16.1 [a]	23.9 [b]	1.6	<0.001	3.1
Stomach weight (mg)	92.9	88.4	99.7	8.3	0.4	16.6
Colon weight with contents (mg)	45.9 [a]	53.5 [b]	53.3 [b]	3.5	0.05	7.0
Colon weight no contents (mg)	35.1	39.3	40.4	2.4	0.07	4.8
Liver weight (mg)	157.6 [a]	178.7[b]	187.5 [b]	8.3	0.002	16.5
Spleen weight (mg)	13.5	15.6	14.8	1.5	0.3	2.9
Kidneys weight (mg)	71.4	75.5	76.8	3.1	0.1	6.3
Brain weight (mg)	347.3 [a]	371.6 [b]	379.0 [b]	6.5	<0.001	12.9
Femur weight (mg)	68.3	65.6	73.1	6.2	0.2	8.5
Visceral fat weight (mg)	81.0	83.0	95.3	10.0	0.3	19.9
Relative organs length and weight per mouse weight						
GIT length (mm g^{-1})	46.0	44.2	44.1	1.3	0.2	2.6

SI length (mm g^{-1})	38.2	35.1	35.0	1.6	0.07	3.1
Colon length plus rectum (mm g^{-1})	8.0	7.5	7.7	0.3	0.2	0.6
Colon length (mm g^{-1})	3.1 [a]	3.3 [a]	4.5 [b]	0.7	<0.001	0.7
Stomach (mg g^{-1})	20.1	17.5	19.1	1.6	0.2	3.2
Colon weight with contents (mm g^{-1})	9.8	10.8	10.2	0.6	0.2	1.3
Colon weight no content (mg g^{-1})	7.2	7.9	7.4	0.6	0.4	1.1
Liver (mg g^{-1})	33.7	34.4	36.0	1.5	0.2	3.0
Spleen (mg g^{-1})	2.8	3.1	2.8	0.3	0.3	0.4
Kidneys (mg g^{-1})	15.3	15.3	14.1	0.6	0.07	1.1
Brain (mg g^{-1})	75.1	75.7	73.3	2.5	0.6	5.0
Femur (mg g^{-1})	16.8	16.1	18.0	1.6	0.4	3.2
Visceral fat weight (mg g^{-1})	16.8	16.1	18.0	1.6	0.4	3.2

Pup at weaning (n= Control, 27; Combo, 27; CMO, 23).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

fed combo and control diets. Absolute brain weight was increased in pups at weaning (control, 346.20; combo, 369.60; CMO, 378.9 ± 6.5; mean [mg] ± SE; P = 0.008) and 30 days after weaning (control, 394.7; combo, 405.5; CMO, 413.9 ± 6.80; mean [mg] ± SE; P =.0.01) (Table 5.7) from dams fed the CMO or combo diets (at weaning only) compared to pups from dams fed the control diet.

After 30 days receiving the control diet, pups from dams fed the CMO diet had increased absolute (control, 716.9; combo, 907.3; CMO, 891.6 ± 68.1; mean [mg] ± SE; P = 0.009) and normalised (control, 45.6; combo, 52.6; CMO, 55.8 ± 4.1; mean [mg/g] ± SE; P = 0.05) visceral fat weight compared to pups from dams that were fed the control diet (Table 5.7). There was no difference in the pups body weight.

5.7.4 Colon histology

Crypt lengths were measured in transverse sections of colon tissue of dams and pups to assess changes in growth of the colonic mucosa. There was no evidence that diet influenced the dams and pups colon crypt lengths (Table 5.8).

The number of mucus containing goblet cells was counted to measure CMO-induced changes in the mucus producing potential of the colonic crypts. No significant differences in goblet cell numbers among diets were observed in dams and pups (Table 5.8).

5.7.5 Leptin and glucose blood concentration

The consumption of CMO or combo diet by the dams did not affect blood glucose (Figure 5.5) and leptin concentration (Table 5.9). 30 days after weaning, pups from dams fed CMO diet, however, had increased concentrations of plasmatic leptin compared to pups from control-fed dams (control, 7.1; combo, 9.5; CMO, 10.4 ±1.3; ng/mL, mean ± SE; P = 0.05) (Table 5.9).

Table 5.7 Absolute and normalised mean body and organ length and weight in pup from control, combo and caprine milk oligosaccharides-fed dams fed control diet for 30 days after weaning.

	Control	Combo	CMO	SE	P value	LSD
Body weight (g)	15.4	16.7	16.4	0.8	0.2	1.7
Body length (cm)	7.7	7.8	7.7	0.1	0.7	0.3
Absolute organs length and weight						
GIT length (mm)	338.1	336.9	341.6	8.2	0.8	16.4
SI length (mm)	282.8	286.5	286.1	5.4	0.7	10.8
Colon length plus rectum (mm)	56.4	55.1	56.8	1.8	0.5	3.6
Colon length (mm)	22.0	21.0	20.8	1.1	0.6	2.2
Stomach weight (mg)	645.8	704.4	711.8	94.2	0.7	189.2
Colon weight with contents (mg)	159.6	160.7	159.6	12.7	0.8	25.6
Colon weight no contents (mg)	87.3	88.6	83.7	5.0	0.6	10.1
Liver weight (mg)	814.9	889.2	836.3	58.4	0.4	58.4
Spleen weight (mg)	52.3	56.9	51.2	3.7	0.2	7.5
Kidneys weight (mg)	207.4	228.1	218.1	11.9	0.2	24.0
Brain weight (mg)	393.7 [a]	404.7 [ab]	413.9 [b]	6.8	0.01	13.6
Femur weight (mg)	113.1	118.3	112.2	6.0	0.5	12.0
Visceral fat weight (mg)	705.9 [a]	881.1 [b]	899.3 [b]	68.1	0.01	136.8
Relative organs length and weight per mouse weight						
GIT length (mm g^{-1})	22.4	20.5	21.3	1.2	0.2	2.4

SI length (mm g^{-1})	18.8	17.4	17.8	1.0	0.3	1.9
Colon length plus rectum (mm g^{-1})	3.7	3.3	3.5	0.2	0.1	0.4
Colon length (mm g^{-1})	1.4	1.3	1.3	0.1	0.1	0.2
Stomach (mg g^{-1})	41.0	45.0	44.7	5.4	0.7	11.1
Colon weight with contents (mm g^{-1})	10.5	10.0	9.5	0.8	0.5	1.6
Colon weight no content (mg g^{-1})	5.7	5.4	5.2	0.3	0.2	0.6
Liver (mg g^{-1})	52.9	52.5	51.7	2.9	0.9	5.8
Spleen (mg g^{-1})	3.4	3.4	3.2	0.3	0.5	0.5
Kidneys (mg g^{-1})	13.5	13.6	13.5	0.7	0.9	1.4
Brain (mg g^{-1})	26.1	24.7	25.8	1.4	0.5	2.8
Femur (mg g^{-1})	7.4	7.1	6.9	0.4	0.4	0.8
Visceral fat weight (mg g^{-1})	46.2 [a]	52.2 [ab]	55.9 [b]	4.1	0.05	8.1

Pup 30 days after weaning (n= Control, 18; Combo, 19; CMO, 16). Values with similar letters in rows do not differ significantly (P<0.05).

[a, b, c] Values with similar letters in rows do not differ significantly (P < 0.05).

Table 5.8 Colon crypt length (μm) and goblet cell numbers in dams (fed control, combo and caprine milk oligosaccharides diet), pups at weaning and pups 30 days after weaning (fed control diet).

		Control	Combo	CMO	SE	P-value	LSD
Crypt	Dams	100	107	114	13	0.5	27
	Pups at weaning	82	90	86	5	0.2	10
	Pups 30 days	84.6	82.8	80.7	3.8	0.6	7.84
Goblet	Dams	14	15	15	2	0.9	5
	Pups at weaning	7.5	9.3	8.7	0.8	0.09	1.6
	Pups 30 days	10.0	10.2	9.4	0.5	0.3	1.13
Goblet/crypt	Dams	0.14	0.14	0.13	0.01	0.6	0.03
	Pups at weaning	0.09	0.1	0.1	0.004	0.1	0.009
	Pups 30 days	0.11	0.12	0.11	0.007	0.3	0.01

Dams (n= Control, 7; Combo, 8; CMO, 7).

Pup at weaning (n= Control, 21; Combo, 24; CMO, 18).

Pup 30 days after weaning (n= Control, 16; Combo, 16; CMO, 14).

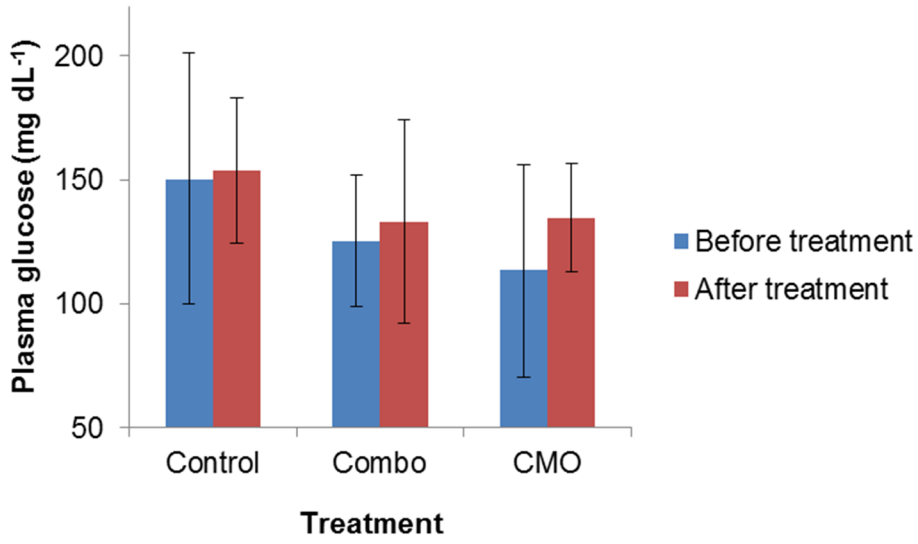

Figure 5.5. Glucose concentration in dam plasma before and after being fed with caprine milk oligosaccharides (CMO), combo or control diet from mating to weaning. Values are means with their standard errors. The same dams were evaluated before and after treatment (n= Control, 7; Combo, 6; CMO, 6).

Table 5.9 Effects of diet on serum leptin in dams and weaned pup (ng/mL).

Group	Control	Combo	CMO	SE	P value	LSD
Dams	14.9	12.8	11.9	2.4	0.4	5.1
Pup	7.1[a]	9.5[ab]	10.4[b]	1.3	0.05	2.8

Dams (n= Control, 7; Combo, 8; CMO, 7).

Pup 30 days after weaning (n= Control, 17; Combo, 16; CMO, 13).

[a, b,] Values with similar letters in rows do not differ significantly (P<0.05).

5.7.6 Milk composition

The effects of diet on dam milk were tested in a pooled sample of dams receiving CMO (5), combo (4) or the control (3) diet. Feeding the CMO and combo diets to the dam resulted in an increase of the crude protein levels in milk (control, 74.8; combo, 115.9; CMO, 115.0 ± 13.30; mg/mL, mean ± SE; $P = 0.02$). There was a trend ($P < 0.1$) for fat concentration in milk to be lower in dams fed the CMO diet compared to dams fed the control diet. There was no evidence; however, that diet changed dry matter, lactose, glucose and galactose concentrations of milk (Table 5.10).

5.7.7 Femur mineral composition

The calcium, magnesium and zinc composition in the dam and pup femurs were analysed. Dams fed the combo diet had higher zinc concentration in the femur compared to control and CMO diets (control, 203.2; combo, 218.8; CMO, 196.4 ± 7.8; µg/g, mean ± SE; $P = 0.02$) (Table 5.11). At weaning, pups from dams fed the combo diet had a higher zinc concentration in the femur (control, 194.4; combo, 214.1; CMO, 189.2 ± 10.8; µg/g, mean ± SE; $P = 0.05$) compared to the pups from dams fed the CMO diet, and a higher magnesium concentration (control, 2.58; combo, 2.75; CMO, 2.62 ± 0.6; µg/g, mean ± SE; $P = 0.02$) when compared to pups from dams fed the control diet (Table 5.11). At weaning, pups from dams fed the combo or CMO diet had a higher calcium concentration in the femur (control, 96.4; combo, 108.4; CMO, 105.7 ± 3.7; µg/g, mean ± SE; $P = 0.004$) when compared to pups from dams fed the control diet. No differences in Ca, Mg and Zn concentration were found among pups 30 days after weaning (Table 5.11).

5.7.8 Caecum SCFA concentrations

Analysis of SCFA concentrations in caecum digesta was carried out by FID capillary GC to measure changes in bacterial metabolism (Table 5.12). After the experimental period (mating to weaning) the caeca of dams fed the combo diet showed higher concentrations of

Table 5.10. Milk nutritional composition (mean ± SD) of mice fed caprine milk oligosaccharides, combo or control diets.

	Control	Combo	CMO	SE	P value	LSD
DM (%)	66.6	70.6	70.4	4.2	0.6	9.5
Total protein (mg/mL)	74.8[a]	115.9[b]	115.0[b]	13.3	0.02	29.6
Total fat (%)	14.7	9.5	5.3	3.0	0.1	9.7
Lactose (mg/mL)	19.1	20.7	22.2	3.5	0.6	7.9
Glucose (mg/mL)	0.5	0.2	0.5	0.1	0.1	0.3
Galactose (mg/mL)	0.5	0.4	0.8	0.1	0.09	0.4

Each sample is a pool of 3 milk samples collected from one animal at lactation days 5, 12 and 19.

Samples (n= Control, 3; Combo, 4; CMO, 5). Different samples number for crude fat analysis (n= Control, 1; Combo, 3; CMO, 2)

[a, b,] Values with similar letters in rows do not differ significantly ($P < 0.05$).

Table 5.11 Effects of diet on femur mineral composition in dams (fed control, combo and caprine milk oligosaccharides diet), pups at weaning and pups 30 days after weaning (fed control diet).

Mineral	Group	Control	Combo	CMO	SE	P-value	LSD
Ca (mg/g)	Dams	195.3	198.9	186.3	8.0	0.3	16.7
	Pups at weaning	96.4[a]	108.4[b]	105.7[b]	3.7	0.004	7.5
	Pups 30 days	187.3	185.6	191.6	5.8	0.5	11.7
Mg (mg/g)	Dams	3.91	4.06	3.81	0.14	0.2	0.29
	Pups at weaning	2.58[a]	2.75 [b]	2.62 [a]	0.6	0.02	0.14
	Pups 30 days	3.87	3.85	3.89	0.96	0.8	1.93
Zn (µg/g)	Dams	203.2[ab]	218.8[a]	196.4[b]	7.8	0.02	16.2
	Pups at weaning	194.4[ab]	214.1[a]	189.2[b]	10.8	0.05	21.6
	Pups 30 days	203.2	188.2	184.3	10.0	0.1	20.2

Dams (n= Control, 7; Combo, 9; CMO, 7).

Pups at weaning (n= Control, 27; Combo, 26; CMO, 22).

Pups 30 days after weaning (n= Control, 17; Combo, 22; CMO, 13).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

propionic (control, 3.1; combo, 5.1; CMO, 3.8 ± 0.4; µmol/g, mean ± SE; P = 0.01) and butyric acids (control, 6.1; combo, 8.6; CMO, 5.5 ± 1.0; µmol/g, mean ± SE; P = 0.01) than the caeca of dams fed the control and CMO diet. Combo and CMO-fed dams, however showed decreased concentrations of caecal isobutyric acid when compared to control-fed dams (control, 0.43; combo, 0.36; CMO, 0.33 ± 0.03; µmol/g, mean ± SE; P = 0.04). CMO-fed dams showed higher concentrations of caecal formic acid when compared to the control fed dams. No significant differences in acetic, lactic and succinic acid concentrations were seen in caecum contents of dams receiving control, combo and CMO diets during the experimental period.

Maternal dietary intervention was also shown to modify the pups SCFA production (Table 5.12). At weaning, pups from CMO-fed dams had an increased concentration of caecal butyric acid (control, 0.7; combo, 1.3; CMO, 2.9 ± 0.4; µmol/g, mean ± SE; P = 0.01) when compared to pups from dams fed control or combo diets. This increase was not observed after 30 days of weaning (Table 5.12). However, 30 days after weaning, pups from the dams fed the combo diet showed an increased concentration of caecal propionic acid (control, 1.3; combo, 2.9; CMO, 2.4 ± 0.6; µmol/g, mean ± SE; P = 0.02) compared to the pups from the dams fed the control diet. There were no significant differences in acetic, lactic and formic acid concentrations in the caecal content of the dams. Isobutyric and succinic acid were not detected in the caecal contents of the pups.

5.7.9 Microbiota composition of dams and pups

The microbiota composition of the colon contents of dams and pups after the experimental period (from mating to weaning) was examined by high throughput 454 pyrosequencing of the bacterial 16S rRNA gene. The total number of reads was 774166 and 671970 sequences with an average product length of 404 and 392 bp from Pool 1 and Pool 2, respectively. The unweighted (Figure 5.7 A and B) and weighted (Figure 5.7 C and D) principal coordinates analysis (PCoA) of dams, pups at weaning and pups 30 days after

Table 5.12 Short chain fatty acids concentrations (µmol/g) in caecum digesta of dams, pups at waning and pups 30 days after weaning.

SCFA	Group	Control	Combo	CMO	SE	P value	LSD
Formic	Dams	0.06[b]	0.16[ab]	0.48[a]	0.15	0.04	0.33
	Pups at weaning	2.0	0.9	1.3	0.5	0.1	1.0
	Pups 30 days	0.7	0.5	0.3	0.2	0.2	0.4
Acetic	Dams	9.0	10.2	9.1	1.2	0.5	2.5
	Pups at weaning	4.4	4.5	4.7	0.7	0.9	1.4
	Pups 30 days	4.8	5.8	6.1	1.3	0.4	2.1
Propionic	Dams	3.1 [a]	5.1[b]	3.8 [a]	0.4	0.01	1.0
	Pups at weaning	1.4	2.2	1.9	0.3	0.1	0.7
	Pups 30 days	1.36[b]	2.92 [a]	2.44[ab]	0.62	0.02	1.27
Butyric	Dams	6.1 [a]	8.6 [b]	5.5 [a]	1.0	0.01	2.2
	Pups at weaning	0.7[b]	1.3[b]	2.9[a]	0.4	0.001	0.9
	Pups 30 days	2.9	4.3	3.9	0.8	0.1	1.7
Lactic	Dams	0.12	0.36	0.09	0.18	0.26	0.39
	Pups at weaning	0.52	0.37	0.27	0.18	0.3	0.36
	Pups 30 days	1.4	1.2	1.5	0.75	0.9	1.5
Isobutyric	Dam	0.43 [b]	0.36 [a]	0.33 [a]	0.03	0.04	0.07
Succinic	Dam	0.13	0.29	0.41	0.14	0.18	0.31

Dams (n= Control, 7; Combo, 9; CMO, 7).

Pups at weaning (n= Control, 20; Combo, 22; CMO, 21).

Pups 30 days after weaning (n= Control, 16; Combo, 11; CMO, 13).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

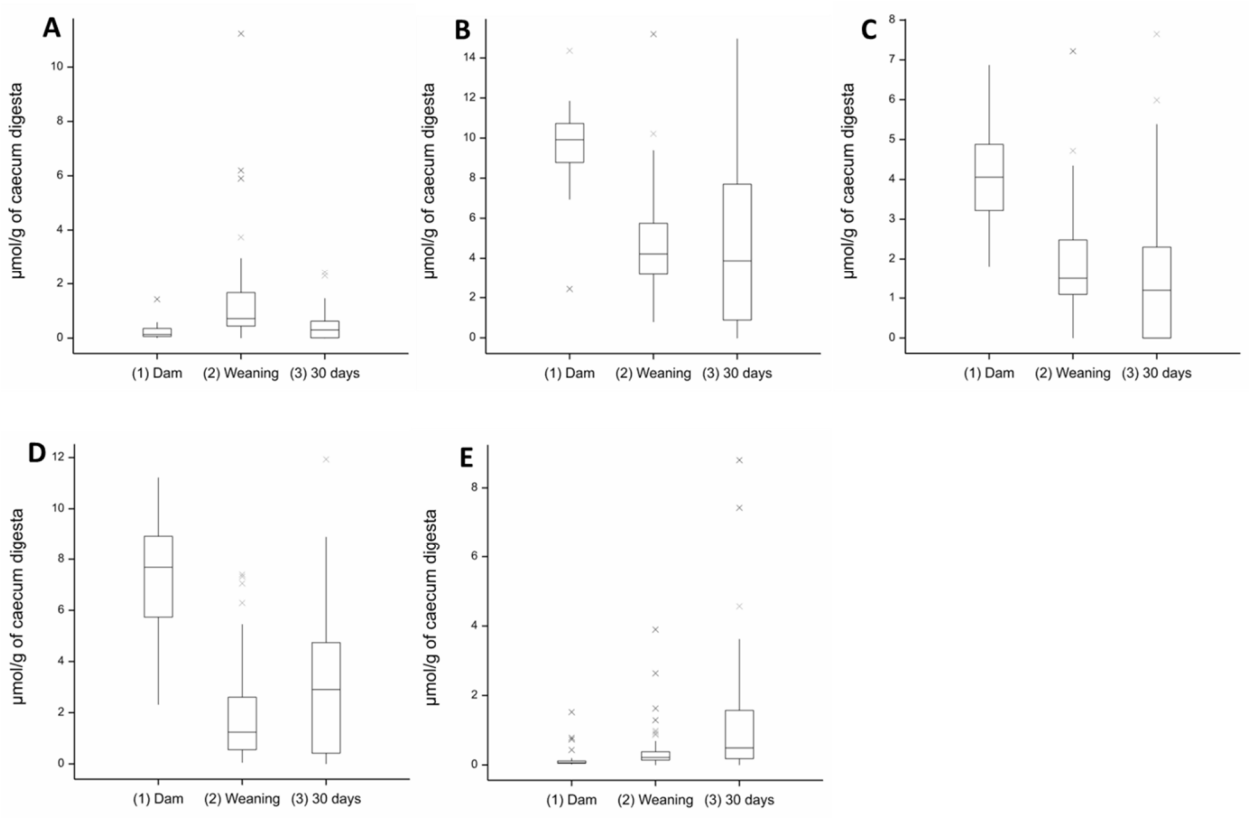

Figure 5.6 Effect of age on caecal short chain fatty acids concentrations measured by flame ionisation detector gas chromatography. Caecal concentrations (µmol/g of caecum digesta) of formic (A), acetic (B), propionic (C), butyric (D) and lactic acid (E) in (1) dams, (2) pups at weaning and (3) pups 30 days after weaning. Band inside of the box represent the median and lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Outliers are plotted as individual points. (n= Dams, 23; Pups at weaning, 63; Pups 30 days after weaning, 40).

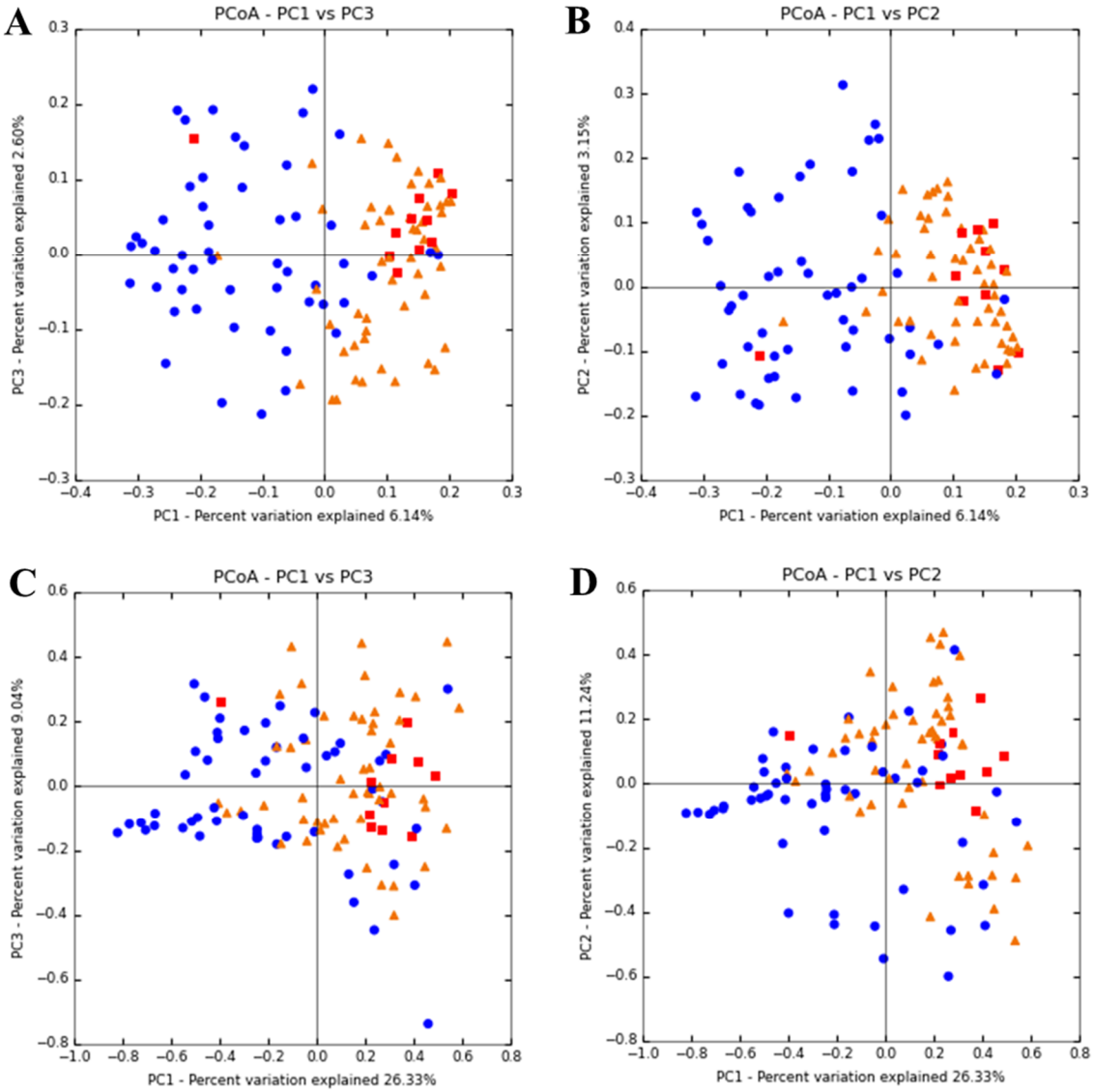

Figure 5.7. PCoA plot of 16S rRNA profiles showing the distances in microbial diversity of colon digesta between dams, pups at weaning and pups 30 days. 16S rRNA unweighted (A and B) weighted (C and D) profiles. Coloured points indicate profile of individual mouse, with colours indicating the bacterial 16S rRNA profiles of dam (red square), pups at weaning (blue circle) and pups 30 days after weaning (orange triangle).

weaning showed different colon microbial community structures. The microbial diversity of colon digesta among treatments was observed in the unweighted PCoA plot (Figure 5.8, A and B). Differences in colon digesta microbial diversity among diet and age groups were also observed in the unweighted PCoA plot (Figure 5.9, A and B). Pups at weaning and pups 30 days after weaning were shown to have higher microbial community diversity when compared to that of the dams (Figure 5.10, A). No significant difference was found among diets for dams and pups (Figure 5.10, B).

Taxonomic classification of sequences using the RDP pyrosequencing pipeline at an 80% confidence threshold showed significant differences in colonic microbiota community composition of the dams and pups after dietary intervention. Low sample numbers of dam colon digesta were amplified and sequenced (n= control, 6; combo, 2; CMO, 2). Pups, however, were better represented by a higher number of samples (weaning (n= control, 19; combo, 20; CMO, 13); pup 30 days after weaning (n= control, 18; combo, 18; CMO, 16)). Summary of the main colonic taxa modulated by diet in dams, pups at weaning and pups 30 days after weaning are presented in Table 5.13, Table 5.14 and Table 5.15. All identified taxa modulated by diet in all age groups are found in full in Appendix A, Appendix B and Appendix C.

Although dietary intervention showed no phylum wide alterations in the dam colon digesta microbiota, proportion of members of a family Porphyromonadaceae were decreased in combo and CMO-fed dams compared to that of the control group (control, 41.3%; combo, 11.9%; CMO, 28.2%) (Table 5.13). At the genus level, the proportions of *Odoribacter* spp., a member of the Porphyromonadaceae family, was increased in the colon digesta of CMO-fed dams compared to that of the combo and control-fed dams (control, 0.4%; combo, 0.6%; CMO, 1.8%). Members of the Clostridia (Unclassified clostridia; control, 0.02%; combo, 0.01%; CMO, 0.06%) such as *Oscillibacter* spp. (control, 0.7%; combo, 0.1%; CMO, 1.5%)

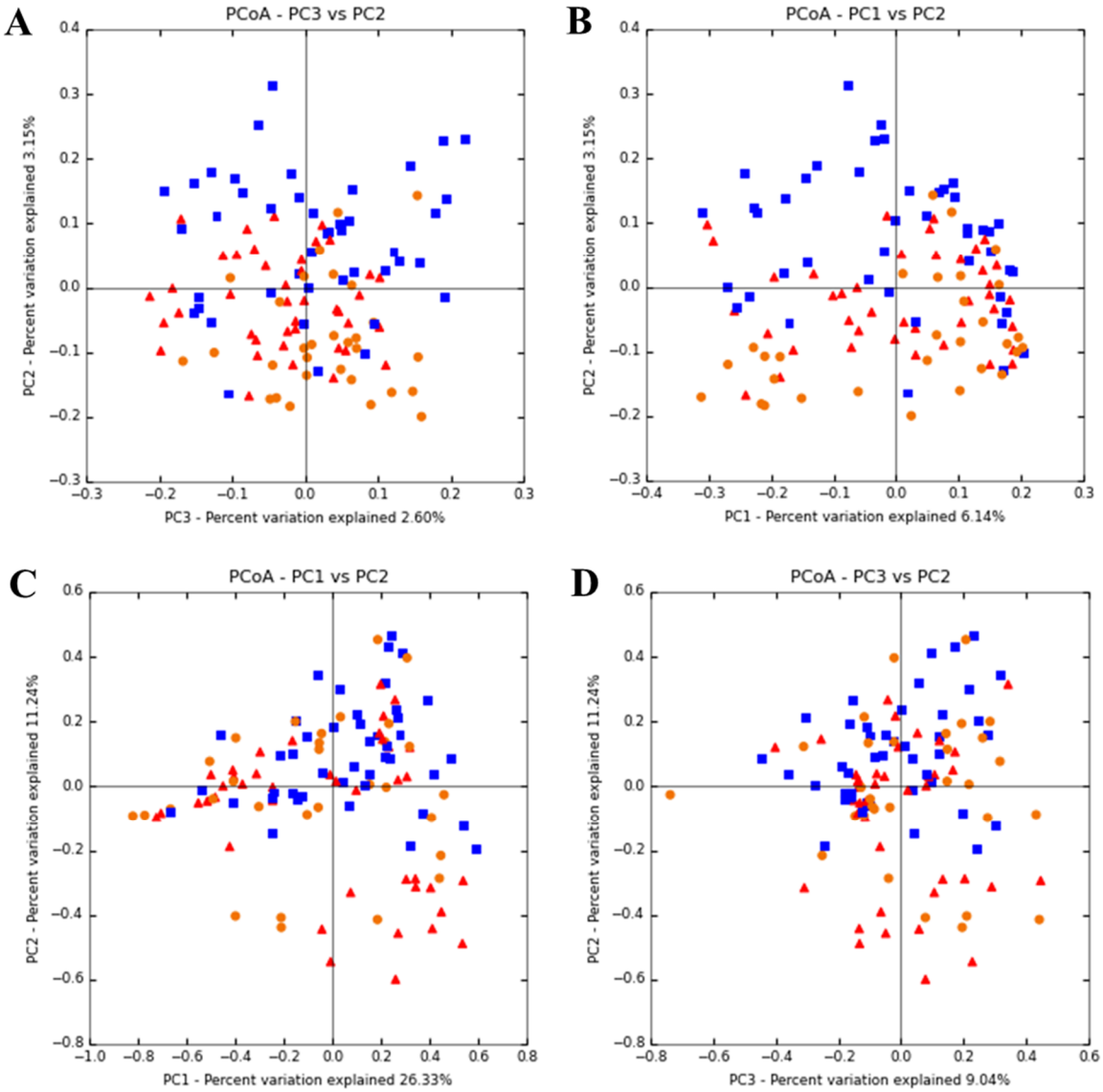

Figure 5.8. PCoA plot of 16S rRNA profiles showing the distances in microbial diversity of colon digesta between treatments. 16S rRNA unweighted (A and B) weighted (C and D) profiles. Coloured points indicate profile of one mouse, with colours indicating the bacterial 16S rRNA profile of control (blue square), combo (red triangle) and Caprine milk oligosaccharide diet (orange circle).

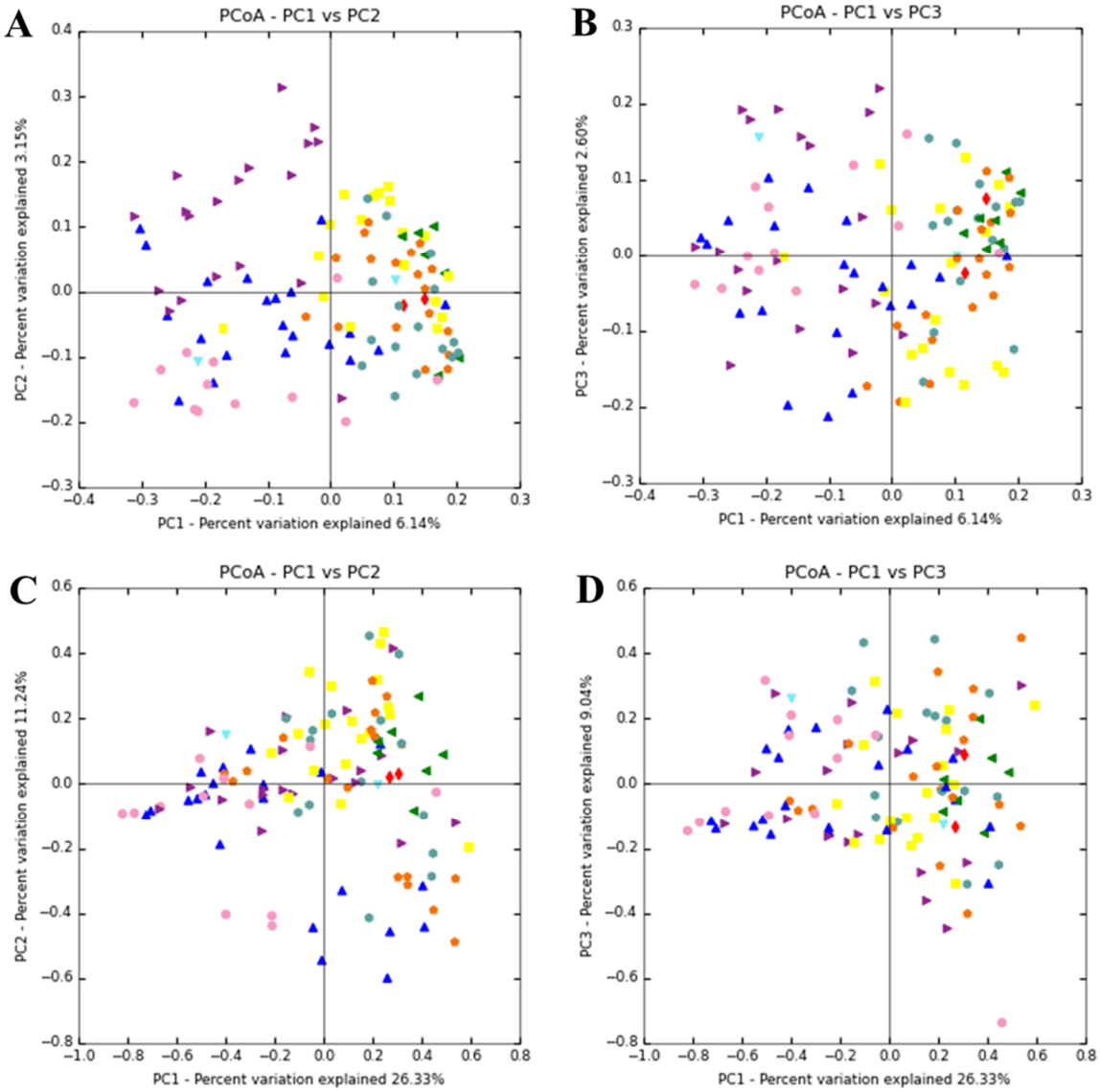

Figure 5.9. PCoA plot of 16S rRNA profiles showing the distances in microbial diversity of colon digesta between dams, pups at weaning and pups 30 days from different treatments. 16S rRNA unweighted (A and B) weighted (C and D) profiles, Coloured points indicate profile of one mouse, with colours indicating the bacterial 16S rRNA profile of dam (control, green triangle; combo, red diamond; caprine milk oligosaccharide (CMO) diet, light blue triangle), pup at weaning (control, purple triangle; combo dark, blue triangle; CMO diet, pink circle) and pup 30 days after weaning (control, yellow square; combo, orange heptagon; CMO diet, light green circle).

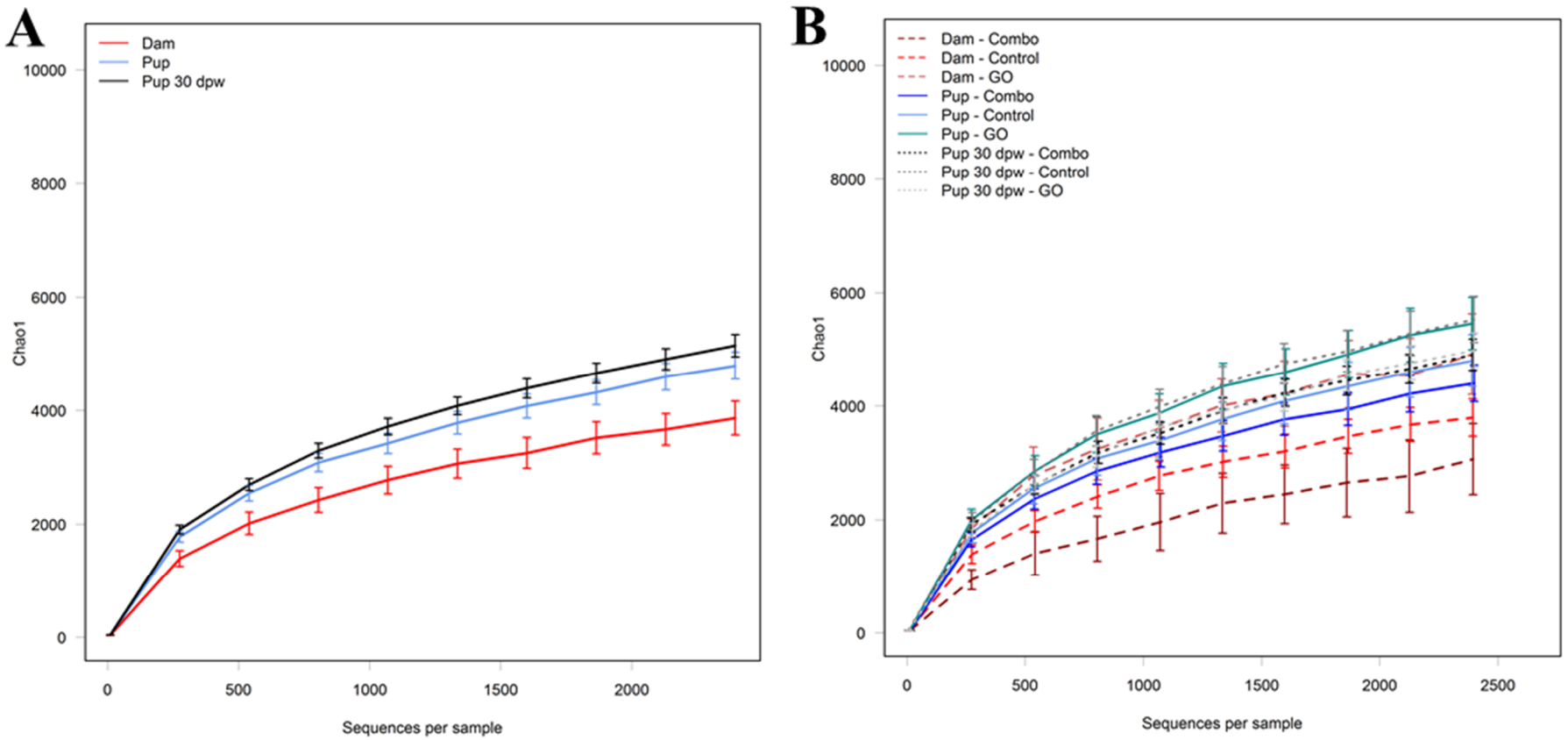

Figure 5.10 Alpha diversity analysis: Rarefaction plot of Chao1 diversity index showing bacterial diversity within samples. (A) Profile of dam (red), pup at weaning (blue) and pup 30 days after weaning (black). (B) Profile of dam (dashed line), pup at weaning (solid line) and pup 30 days after weaning (dotted line) accordingly with treatment.

Table 5.13 Summary of the main colonic taxa modulated by diet in dams. Values are the percentage of 16S rRNA sequences present in the colon digesta.

Depth	Identification	Control	Combo	CMO	P-value	FDR
Phylum	Tenericutes	0.13	0.93	0.65	0.18	1.00
Family	Porphyromonadaceae	41.39[a]	11.97[b]	28.28[b]	0.06	0.55
	Ruminococcaceae	3.96	1.06	5.59	0.12	0.55
	Unclassified Proteobacteria	0.29	0.63	1.30	0.12	0.55
	Unclassified Clostridia	0.02	0.01	0.06	0.16	0.55
	Unclassified Firmicutes	0.24	0.41	0.17	0.18	0.55
	Mycoplasmataceae	0.10	0.93	0.61	0.18	0.55
	Bacteroidaceae	10.74	35.17	2.78	0.21	0.55
	Erysipelotrichaceae	0.52	4.25	2.37	0.21	0.55
	Rikenellaceae	5.30	2.31	13.58	0.21	0.55
Genus	Odoribacter	0.45[a]	0.62[a]	1.83[b]	0.02	0.54
	Lactococcus	0.02[a]	0.14[b]	0.05[a]	0.05	0.54
	Unclassified Porphyromonadaceae	38.17[a]	10.70[b]	22.14[b]	0.05	0.54
	Oscillibacter	0.70[a]	0.11[a]	1.52[b]	0.07	0.54
	Unclassified Clostridia	0.02[a]	0.01[a]	0.06[b]	0.07	0.54
	Allobaculum	0.27[a]	3.89[b]	1.97[ab]	0.08	0.54
	Ureaplasma	0.08	0.92	0.61	0.11	0.54
	Bifidobacterium	0.01	0.12	0.00	0.12	0.54
	Unclassified Erysipelotrichaceae	0.03	0.19	0.20	0.13	0.54

Robinsoniella	0.23	1.04	2.31	0.14	0.54
Unclassified Coriobacteriaceae	0.00	0.01	0.02	0.15	0.54
Sporacetigenium	0.58	3.30	0.69	0.15	0.54
Unclassified Peptostreptococcaceae	0.68	4.57	1.17	0.16	0.54
Ethanoligenens	0.01	0.00	0.05	0.16	0.54
Unclassified Proteobacteria	0.29	0.63	1.30	0.16	0.54
Unclassified Firmicutes	0.24	0.41	0.17	0.17	0.54
Bacteroides	10.74	35.17	2.78	0.18	0.54
Alistipes	5.07	2.18	12.89	0.22	0.63

Dams (n= Control, 6; Combo, 2; CMO, 2).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

FDR, false discovery rate.

Table 5.14 Summary of the main colonic taxa modulated by diet in the pups at weaning. Values are the percentage of 16S rRNA sequences present in the colon digesta.

Depth	Identification	Control	Combo	CMO	P-value	FDR
	Unclassified Epsilonproteobacteria	0.000	0.001	0.002	0.07	0.96
	Carnobacteriaceae	0.000	0.003	0.000	0.14	0.96
	Unclassified Desulfovibrionales	0.62	0.37	0.98	0.14	0.96
Family	Desulfovibrionaceae	0.05	0.02	0.06	0.14	0.96
	Unclassified Bacillales	0.000	0.006	0.000	0.14	0.96
	Staphylococcaceae	0.04	0.17	0.02	0.16	0.96
	Unclassified Proteobacteria	0.95	0.59	1.66	0.22	0.96
	Bifidobacterium	0.05 [a]	0.07 [a]	1.37 [b]	0.03	0.86
	Unclassified Bifidobacteriaceae	0.01 [a]	0.01 [a]	0.13 [b]	0.04	0.86
	Parabacteroides	1.51 [a]	0.48 [a]	4.51 [b]	0.04	0.86
	Barnesiella	3.34 [a]	1.36 [b]	2.18 [ab]	0.05	0.86
	Unclassified Epsilonproteobacteria	0.0000	0.0007	0.002	0.07	0.86
	Unclassified Ruminococcaceae	2.38	3.93	3.33	0.10	0.86
	Alistipes	7.37	7.24	13.05	0.11	0.86
	Unclassified Erysipelotrichaceae	1.36	1.20	3.98	0.12	0.86
	Coprobacillus	0.06	0.72	0.44	0.12	0.86
	Anaerosporobacter	0.001	0.0003	0.003	0.12	0.86
	Unclassified Bacteria	1.39	1.22	1.82	0.13	0.86
Genus	Granulicatella	0.0004	0.003	0.00	0.14	0.86

Unclassified Streptococcaceae	0.0004	0.002	0.003	0.15	0.86
Unclassified Helicobacteraceae	0.04	0.08	0.04	0.17	0.86
Anaeroplasma	0.01	0.04	0.01	0.18	0.86
Roseburia	0.21	0.71	0.30	0.18	0.86
Streptococcus	0.03	0.13	0.02	0.19	0.86

Pup weaning (n= Control, 19; Combo, 20; CMO, 13).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

FDR, false discovery rate.

Table 5.15 Summary of the main colonic taxa modulated by diet in the pups 30 days after weaning. Values are the percentage of 16S rRNA sequences present in the colon digesta.

Depth	Description	Control	Combo	CMO	P-value	FDR
Family	Incertae Sedis XIV	0.001 [a]	0.012 [b]	0.001 [a]	0.0001	0.01
	Peptostreptococcaceae	0.58 [a]	4.67 [b]	1.13 [a]	0.005	0.12
	Clostridiaceae	0.32 [a]	0.73 [b]	0.19 [a]	0.03	0.44
	Rikenellaceae	9.05 [a]	7.49 [ab]	5.60 [b]	0.04	0.49
	Streptococcaceae	0.16	0.24	0.10	0.12	0.76
	Enterobacteriaceae	0.08	0.31	0.08	0.22	0.76
Genus	Blautia	0.0004 [a]	0.01 [b]	0.00 [a]	0.001	0.08
	Turicibacter	0.09 [a]	1.69 [b]	0.12 [a]	0.007	0.26
	Unclassified Peptostreptococcaceae	0.32 [a]	2.71 [b]	0.64 [a]	0.01	0.26
	Sporacetigenium	0.26 [a]	1.96 [b]	0.49 [ab]	0.01	0.26
	Unclassified Firmicutes	0.58 [ab]	0.38 [a]	0.94 [b]	0.01	0.26
	Unclassified Clostridiaceae	0.16 [a]	0.36 [b]	0.08 [a]	0.02	0.29
	Alistipes	8.23 [a]	7.11 [ab]	5.15 [b]	0.04	0.78
	Clostridium	0.17 [a]	0.37 [b]	0.10 [a]	0.04	0.49
	Allobaculum	5.56 [a]	3.09 [a]	10.78 [b]	0.05	0.54
	Barnesiella	3.99 [a]	1.80 [b]	1.88 [b]	0.07	0.58
	Parabacteroides	6.32 [a]	1.55 [b]	1.71 [b]	0.07	0.58
	Unclassified Lactobacillaceae	0.36	0.16	0.15	0.11	0.66
	Lactobacillus	1.22	0.50	0.66	0.12	0.66

Lactococcus	0.10	0.21	0.08	0.13	0.66

Pup 30 days after weaning (n= Control, 18; Combo, 18; CMO, 16).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

FDR, false discovery rate.

were also more abundant in the colon digesta of CMO-fed dams compared to that of the combo and control-fed dams. Other members of the Firmicutes family, *Lactococcus* spp, (control, 0.02%; combo, 0.14%; CMO, 0.05%), and the *Allobaculum* spp. (control, 0.3%; combo, 3.9%; CMO, 1.9%) were found in higher proportions in the colon digesta of combo-fed dams compared to that of the control and CMO-fed dams.

At weaning, pups from dams fed the CMO diet showed genus alterations in the colon digesta community compared to pups from control-fed dams, with significantly higher proportions of *Parabacteroides* spp. (control, 1.5%; combo, 0.5%; CMO, 4.5%), *Bifidobacterium* spp. (control, 0.05%; combo, 0.07%; CMO, 1.37%) and unclassified members of Bifidobacteriacea (control, 0.01%; combo, 0.01%; CMO, 0.1%) (Table 5.14). At weaning, pups from dams fed the combo diet had a significant decrease in proportions of *Barnesiella* spp. in the colon digesta compared to pups from control-fed dams (control, 3.3%; combo, 1.3%; CMO, 2.1%).

Greatest alterations in the colon digesta community were observed in pups 30 days after weaning (Table 5.15). 30 days after weaning, families such as Incertae Sedis XIV (control, 0.001%; combo, 0.1%; CMO, 0.001%), Peptostreptococcaceae (control, 0.6%; combo, 4.6%; CMO, 1.1%) and Clostridiaceae (control, 0.3%; combo, 0.7%; CMO, 0.1%) all members of the Clostridia class were highly represented in pups from dams fed the combo diet compared to the control and CMO diets. Changes in the proportions of Clostridia class were attributed to decreases in *Sporacetigenium* spp. (control, 0.2%; combo, 1.9%; CMO, 0.5%), Clostridium spp (control, 0.1%; combo, 0.3%; CMO, 0.1%), unclassified Clostridiaceae (control, 0.1%; combo, 0.3%; CMO, 0.08%) and Peptostreptococcaceae (control, 0.3%; combo, 2.7%; CMO, 0.6%). 30 days after weaning, the proportions of *Turicibacter* spp., a genus member within Firmicutes, were increased in pups from dams fed combo diet compared to pups from the dams fed the control and CMO diets (control, 0.09%; combo, 1.7%; CMO, 0.1%), whereas *Barnesiella* spp. (control, 3.9%; combo, 1.8%; CMO,

1.8%) and *Parabacterioides* spp. (control, 6.3%; combo, 1.5%; CMO, 1.7%), both members of the Bacteriodetes phylum, were decreased in the pups from dams fed the combo and CMO diets compared to pups from the dams fed the control diet. Proportions of *Allobaculum* spp. was increased in pups from CMO-fed dams when compared to the pups from control and combo-fed dams (control, 5.5%; combo, 3.0%; CMO, 10.7%), 30 days after weaning.

5.7.10 Metabolite profiles of dams and pups

Partial least-square discriminant analysis (PLS-DA), implemented in MetaboAnalyst, was performed to discriminate the metabolite profiles between dams and pups assigned to the different dietary treatments. Applying data filtering using estimate interquantile range reduced 40% of the variables. Autoscaling (mean-centered and divided by standard deviation of each variable) and the statistical methods ANOVA, false discovery rate (FDR) and Fisher's least significant difference method (Fisher's LSD) were applied. Metabolites considered significant at $P \leq 0.005$ or listed as important features identified by PLS-DA to discriminate treatments were identified.

5.7.10.1 Urine

PLS-DA analysis of the processed metabolite data from urine, in both columns and modes, from dams before treatment (Figure 5.11) did not show a significant clustering among the dams assigned to the three dietary treatments, with exception of the metabolites described in Table 5.16. The concentration of the metabolite corresponding to diacylglycerol (M_{LCMS} 346.2) were higher in combo and CMO-fed dams. PLS-DA analysis of urinary metabolites from dams after treatment showed better clustering among the treatments and a clear separation between the metabolites found in the CMO and combo-fed dams (Figure 5.12). Although, PLS-DA analysis of urinary metabolites from pups 30 days after weaning did not show a clear distinction (Figure 5.13), discriminant metabolites among pups from dams fed different diets were identified. Summary of the main dam and pup metabolites modulated by

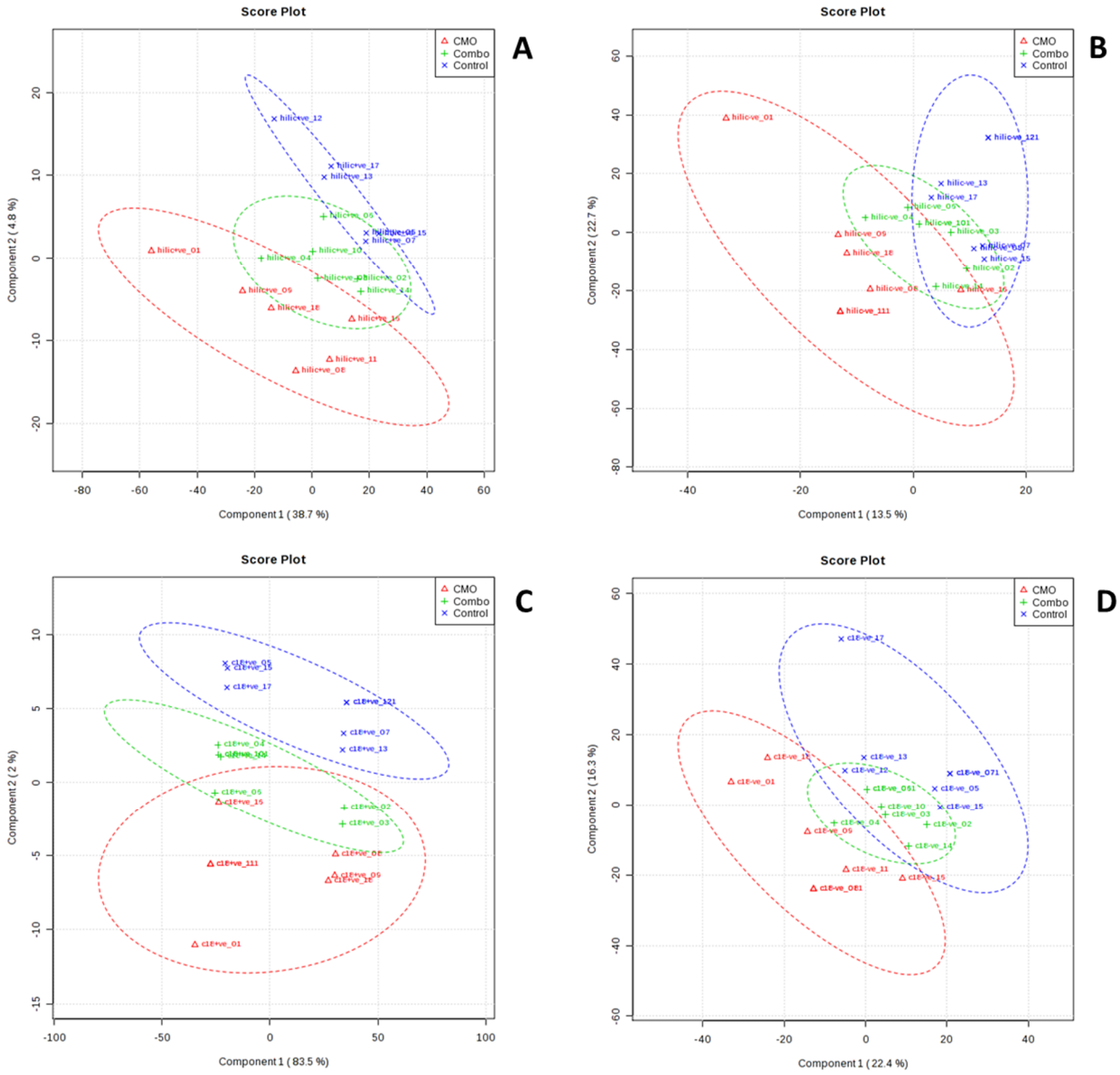

Figure 5.11 The partial least square discriminant analysis (PLS-DA) cross-validated score plot generated from mass spectrometry spectra of urine samples from dams before treatment. control (+), combo (x) and caprine milk oligosaccharides (∆) diet. Samples were run in two different columns HILIC and C18 and in both positive and negative ionisation modes separately. (A) HILIC +ve; (B) HILIC -ve; (C) C18 +ve; (D) C18 –ve.

Table 5.16 Annotated urine metabolites from liquid chromatography-mass spectrometry analysis in HILIC and C18 columns negative and positive mode found to separate dams before treatment.

Column/Mode	MLCMS [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
HILIC / -	143.0704	0.001	0.9	↑ Combo X Control; CMO	P-cresol	M+Cl	143.0269
HILIC / -	190.0503	0.005	0.9	↑ Combo; CMO X Control	5-Hydroxyindoleacetic acid	M-H	190.0510
HILIC / +	367.172	0.005	0.9	↑ CMO X Control; Combo	11-Dehydrocorticosterone	M+Na	367.1880
C18 / -	346.2233	0.0008	0.9	↑ Combo; CMO X Control	Diacylglycerols	M+2H	346.2716

FDR, false discovery rate.

↑ Combo X Control; CMO – increased presence of adduct in combo treatment compared to control and CMO treatments.

↑ Combo; CMO X Control - increased presence of adduct in combo and CMO treatments compared to control treatment.

↑ CMO X Control; Combo - increased presence of adduct in CMO treatment compared to control and combo treatments.

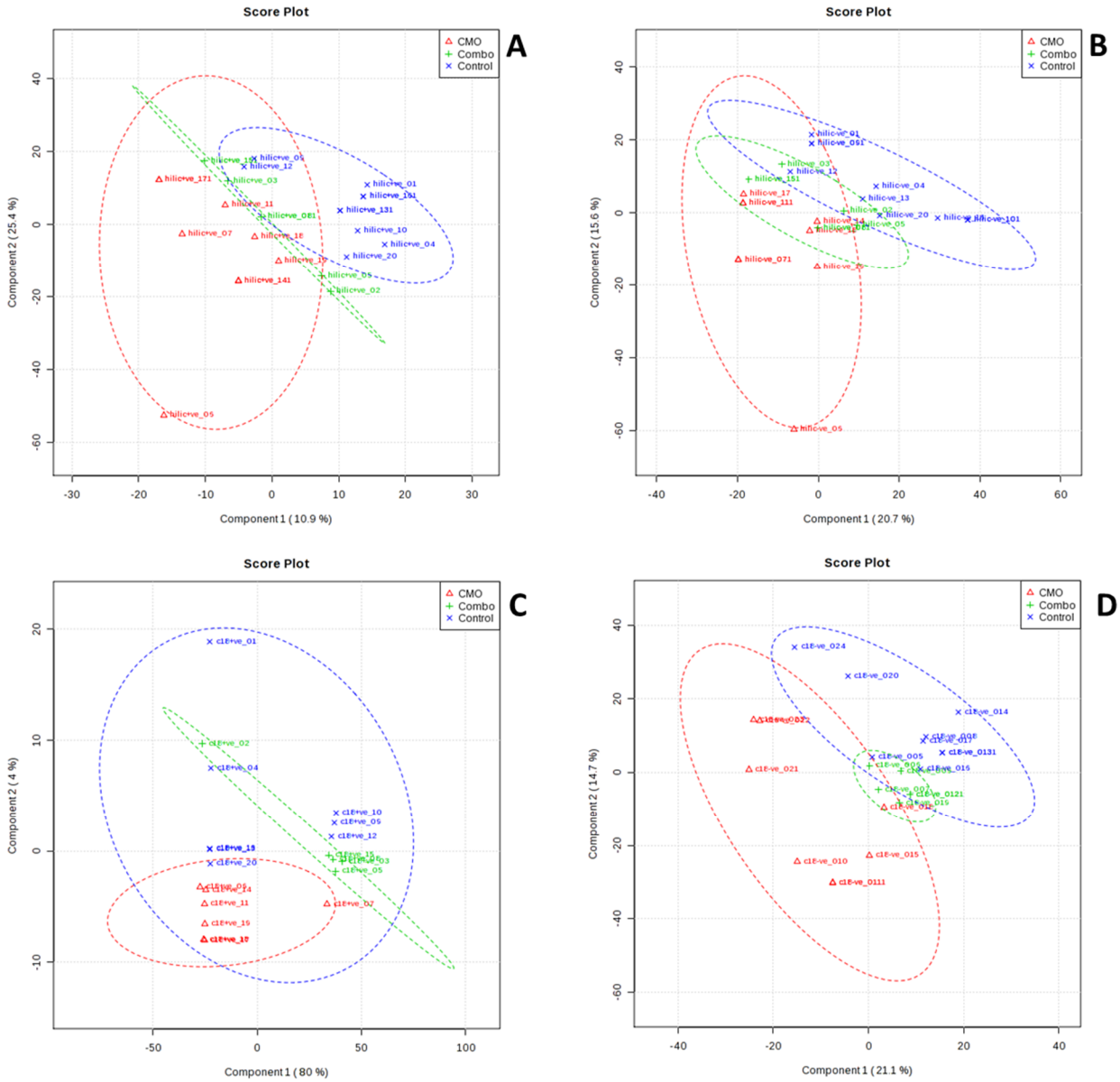

Figure 5.12 The partial least square discriminant analysis (PLS-DA) cross-validated score plot generated from mass spectrometry spectra of urine samples from dams treated with control (+), combo (x) and caprine milk oligosaccharides (Δ) diet. Samples were run in two different columns HILIC and C18 and in both positive and negative ionisation modes separately. (A) HILIC +ve; (B) HILIC -ve; (C) C18 +ve; (D) C18 –ve.

Table 5.17 Summary of urine metabolites from liquid chromatography-mass spectrometry analysis in HILIC and C18 columns negative and positive mode found to separate dams after the treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
Bile acids or liver derivatives							
C18/+	213.1479	0.002	0.1	↑ Combo X Control; CMO	Tetrahydroxy-5b-cholanoic acid	M+2H	213.1485
C18/+	394.3270	0.004	0.1	↑ Combo X Control; CMO	12b-Hydroxy-5b-cholanoic acid	M+NH4	394.3315
					Allolithocholic acid	M+NH4	394.3315
					Lithocholic acid	M+NH4	394.3315
C18/+	477.3647	0.0003	0.1	↑ Combo X Control; CMO	27-Norcholestanehexol	M+Na	477.3186
C18/+	155.1405	0.001	0.1	↑ Combo X Control; CMO;	1-Methylnicotinamide	M+NH4	155.1053
Fatty acids and derivatives							
C18/+	119.0838	0.006	0.1	↑ Combo X Control; CMO	2-Hydroxy-3-methylbutyric acid	M+H	119.0921
C18/+	346.2695	0.001	0.1	↑ Combo X Control; CMO	Docosahexaenoic acid	M+NH4	346.2740
C18 / -	346.2233	0.0008	0.9	↑ Combo; CMO X Control	Diacylglycerols	M+2H	346.2716
C18/+	153.1250	0.005	0.1	↑ Combo X Control; CMO	L-Octanoylcarnitine	M+H+NH4	153.1253
HILIC/ +	736.5043	0.02	*	↑ Combo X Control; CMO	glycerophospholipid	M+H	
HILIC/ +	303.1772	0.03	*	↓ Combo X Control; CMO	Linoleic acid	M+Na	303.2294
C18/+	337.3285	0.006	0.1	↓ Combo X Control; CMO	Palmitoleic acid	M+2ACN+H	337.2849
HILIC/ +	544.7291	0.03	*	↑ CMO X Control; Combo	glycerophospholipid	M+H	

C18/+	179.1394	0.001	0.1	↑ CMO X Control; Combo	L-Carnitine	M+NH4	179.1390
Adrenal metabolites							
HILIC/ +	293.0629	0.003	0.9*	↑ Combo X Control; CMO	Estrone	M+Na	293.1512
C18/+	308.2937	0.0006	0.1	↑ Combo X Control; CMO	Epiandrosterone	M+NH4	308.2584
C18/+	331.2077	0.007	0.1*	↑ Combo X Control; CMO	17-Hydroxyprogesterone	M+H	331.2267
C18/+	348.2769	0.002	0.1	↑ Combo X Control; CMO	17-Hydroxyprogesterone	M+NH4	348.2533
C18/+	311.2543	0.007	0.1*	↑ CMO X Combo	16b-Hydroxyestradiol	M+Na	311.1617
					17-Epiestriol	M+Na	311.1617
Metabolites involved in oxidative stress							
HILIC/ +	101.0711	0.05	*	↑ Combo X Control; CMO	Hexanal	M+H	101.0961
C18/-	164.0400	0.004	0.9	↓ Combo; CMO X Control	Methionine sulfoxide	M-H	164.0386

* Metabolite markers based on the VIP scores of PLS-DA.

↑ or ↓ Combo X Control; CMO – increased or decreased presence of adduct in combo treatment compared to control and CMO treatments.

↑ or ↓ Combo; CMO X Control - increased or decreased presence of adduct in combo and CMO treatments compared to control treatment.

↑ CMO X Control; Combo - increased presence of adduct in CMO treatment compared to control and combo treatments.

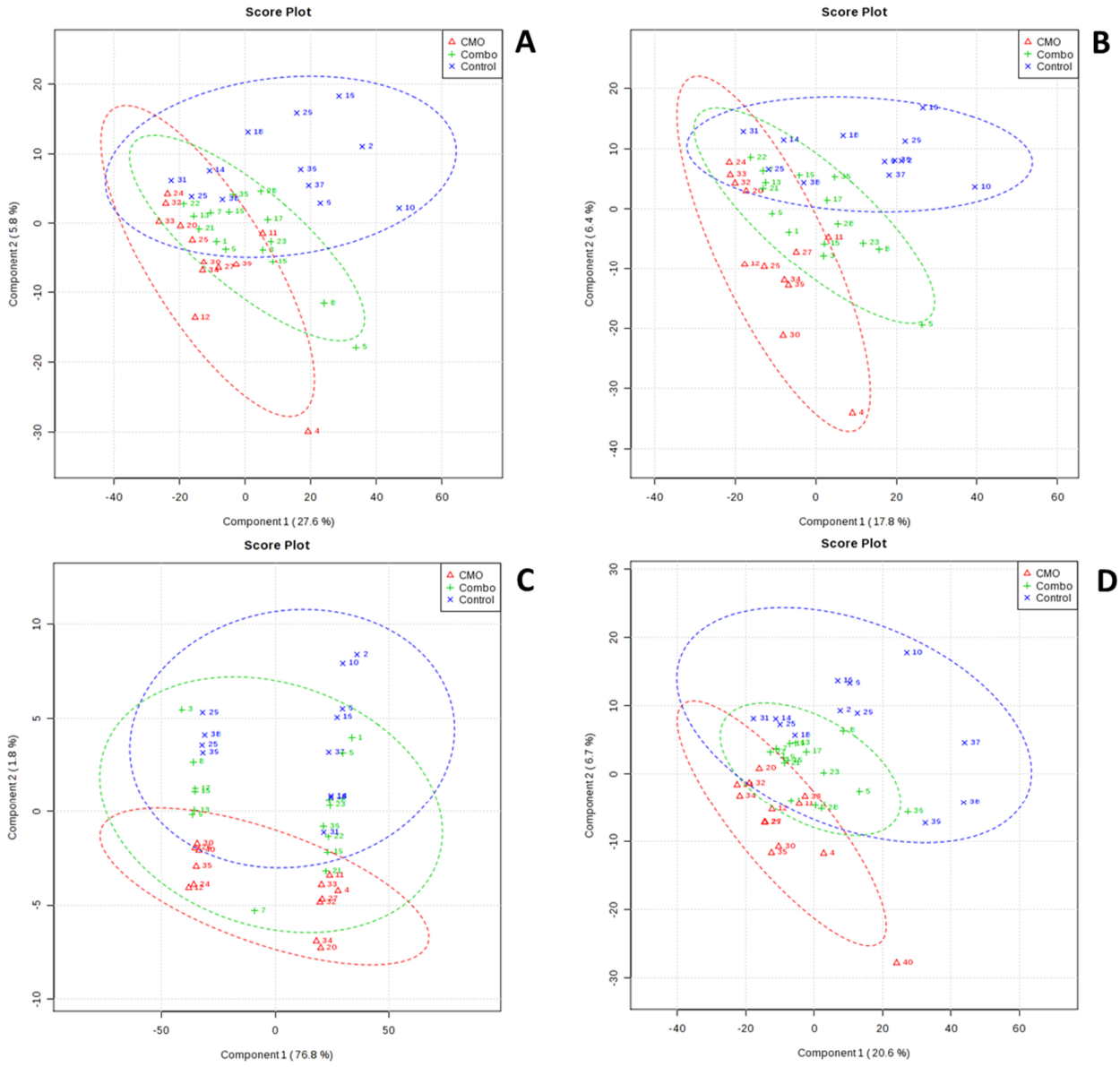

Figure 5.13 The partial least square analysis discriminant analysis (PLS-DA) cross-validated score plot generated from mass spectrometry spectra of urine samples from pups 30 days after weaning from dams treated with control (+), combo (x) and caprine milk oligosaccharides (Δ) diet. Samples were run in two different columns HILIC and C18 and in both positive and negative ionisation modes separately. (A) HILIC +ve; (B) HILIC -ve; (C) C18 +ve; (D) C18 –ve.

Table 5.18 Summary of urine metabolites from liquid chromatography-mass spectrometry analysis, HILIC and C18 columns, negative and positive mode found to separate pups 30 days after weaning accordingly with dams treatment.

Column/Mode	MLCMS [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
Adrenal metabolites							
HILIC/+	308.2216	0.006	*	↑ Combo X CMO	Epiandrosterone	M+NH4	308.2584
					Etiocholanolone	M+NH4	308.2584
HILIC/+	337.2122	0.02	*	↑ Combo X CMO	7-Dehydropregnenolone	M+Na	337.2138
Protein degradation and derivatives							
HILIC/-	160.0606	0.006	0.9*	↑ Combo X Control; CMO	Aminoadipic acid	M-H	161.0688
HILIC/-	219.9999	0.002	0.9	↑ Combo X Control; CMO	O-Phosphohomoserine	M+Na-2H	219.9992
					O-Phosphothreonine	M+Na-2H	219.9992
HILIC/+	291.0443	0.02	*	↑ Combo X CMO	Argininosuccinic acid	M+H	291.1299
C18/-	92.0562	0.003	0.8	↓ CMO X Control; Combo	Phenol	M-H	94.0419
C18/-	107.0477	0.0003	0.5	↓ CMO; Combo X Control	p-Cresol	M-H	108.0575
HILIC/-	196.0722	0.003	0.9	↓ CMO X Control; Combo	Citrulline	M+Na-2H	196.0704
HILIC/+	263.0872	0.001	0.6	↓ CMO; Combo X Control	L-beta-aspartyl-L-glutamic acid	M+H	263.0874
Fatty acids							
HILIC/+	694.4949	0.01	*	↑ Combo X CMO	Glycerophospholipid		
C18/-	85.0638	0.004	0.8	↓ CMO; Combo X Control	But-2-enoic acid	M-H	85.0295
C18/-	107.0477	0.0003	0.5	↓ CMO; Combo X Control	(R)-3-Hydroxydodecanoic acid	M-2H	107.079
C18/-	107.0477	0.0003	0.5	↓ CMO; Combo X Control	3-Oxodecanoic acid	M-2H	58.0419
Metabolites involved in oxidative stress							

C18/-	164.04	0.004	0.9	↓ CMO; Combo X Control	Methionine sulfoxide	M-H	164.0387

* Metabolite markers based on the VIP scores of PLS-DA.

↑ Combo X CMO – increased presence of adduct in combo treatment compared to CMO treatment.

↑ Combo X Control; CMO – increased presence of adduct in combo treatment compared to control and CMO treatments.

↓ Combo; CMO X Control - decreased presence of adduct in combo and CMO treatments compared to control treatment.

↑ CMO X Control; Combo - increased presence of adduct in CMO treatment compared to control and combo treatments.

diet are described in Table 5.17 and Table 5.18. Full description of metabolites is described in Appendix D and Appendix E. Urine of dams fed the combo diet showed an increase in the concentrations of bile acids such as lithocholic acid, 27-norcholestanehexol and tetrahydroxy-5b-cholanoic acid (Table 5.17). Fatty acids concentrations such as docosahexaenoic acid (Omega 3) and 2-hydroxy-3-methylbutiric acid were also increased in the urine of dams fed the combo diet; however, other fatty acids concentrations such as linoleic acid (omega 6) and palmitoleic acid were decreased when compared to CMO and control-fed dams. CMO-fed dams, had an increase in carnitine concentrations, a metabolite involved with fatty acid metabolism, compared to combo and control-fed dams. Increased concentrations of adrenal metabolites were observed in combo-fed dams (estrone, epiandrosterone, ethiocholanone, hydroxiprogesterone) and CMO-fed dams (hydroxyestradiol, epiestriol) compared to the control-fed dams. The same adrenal effect, i.e. increased in epiandrosterone and etiocholanolone, was observed in pups from the dams fed combo diet, 30 days after weaning (Table 5.18). The concentration of metabolites involved in protein degradation were increased in pups from combo-fed dams (aminoadipic acid and o-phosphohomoserine) but decreased in pups from CMO-fed dams (P-cresol, phenol and citrulline), 30 days after weaning. Methionine sulfoxide, a maker for oxidative stress was decreased in pups 30 days after weaning from combo and CMO-fed dams compared to the control group.

5.7.10.2 Plasma

PLS-DA plots of plasma metabolites from dams, obtained from a C18 column in negative mode, did not show any effects of dietary treatment. HILIC (both modes) and C18 positive mode PLS-DA plots showed a clear clustering and discrimination among metabolites present in control, combo and CMO-fed dams (Figure 5.14). A summary of important discriminating metabolites are described in Table 5.19. Full descriptions of metabolites are presented in Appendix F. Plasma of dams fed the CMO diet had an increase in the metabolite

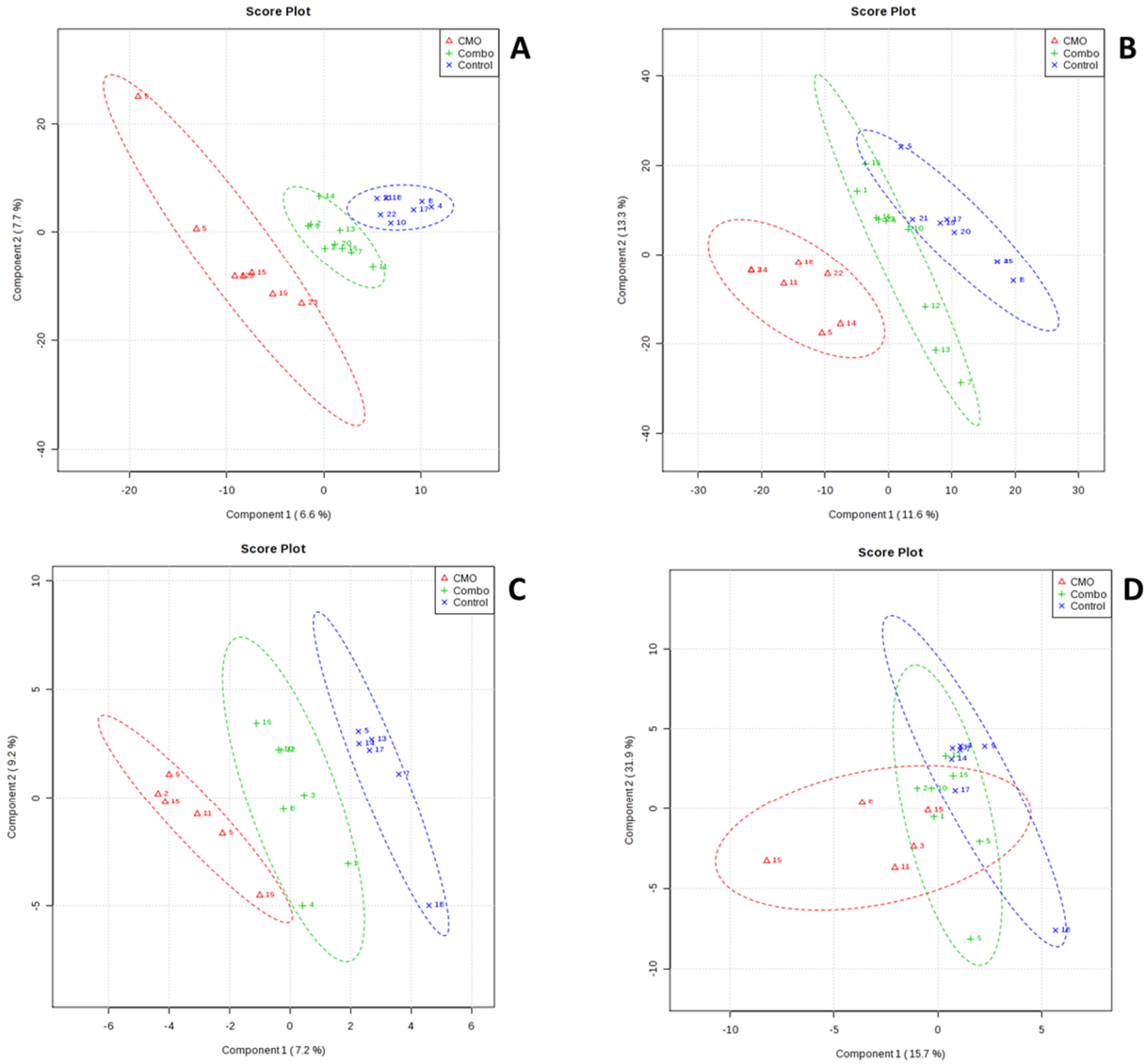

Figure 5.14 The partial least square analysis discriminant analysis (PLS-DA) cross-validated score plot generated from mass spectrometry spectra of plasma samples from dams treated with control (+), combo (x) and caprine milk oligosaccharides (Δ) diet. Samples were run in two different columns HILIC and C18 and in both positive and negative ionisation modes separately. (A) HILIC +ve; (B) HILIC -ve; (C) C18 +ve; (D) C18 –ve.

Table 5.19 Summary of plasma metabolites from liquid chromatography-mass spectrometry analysis, HILIC and C18 columns, negative and positive mode, found to separate dams according to the treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
Fatty acids and/ or mitochondrial derivatives							
HILIC/ +	160.0970[#]	8E-05	0.08	↑ CMO X Control; Combo	3-Dehydrocarnitine	M+H	160.0968
HILIC/ +	205.1550	2E-03	0.7*	↑ CMO X Control; Combo	N-Heptanoylglycine	$M+NH_4$	205.1546
HILIC/ -	471.7480	5E-09	1.3E-06	↑ CMO X Control; Combo	Lactosylceramide (d18:1/22:0)	M-2H	471.8485
					cis,cis-3,6-Dodecadienoyl-CoA	M-2H	471.6182
HILIC/ -	295.6899	1E-08	2.0E-06*	↑ CMO X Control; Combo	Ceramide (d18:1/20:0)	M-2H	295.78007
HILIC/ -	298.6857[#]	2E-08	2.9E-06	↑ CMO X Control; Combo	Triglicerides	M-3H	298.6045
HILIC/ -	393.8184[#]	3E-08	3.7E-06	↑ CMO X Control; Combo	Phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine	M-2H	
HILIC/ -	476.7465	4E-08	5.1E-06	↑ CMO X Control; Combo	Cardiolipins	M-3H	476.671
HILIC/ -	391.8210	8E-08	8.5E-06	↑ CMO X Control; Combo	Phosphatidylethanolamines	M-2H	391.80764
					Sphingomyelin (d16:1/24:1(15Z))	M-2H	391.81955
					Phosphatidylcholines		
HILIC/ -	292.6916	8E-08	8.5E-06	↑ CMO X Control; Combo	Glutaryl-CoA	M-3H	292.70836
					(S)-Hydroxyhexanoyl-CoA	M-3H	292.72049
C18/ +	544.3000[# $]	5E-02	0.5*	↑ Combo - Control	Lysophospholipids	M+Na	544.3374
Bile acids or liver derivatives							
HILIC/ +	160.0970[#]	8E-05	0.08	↑ CMO X Control; Combo	1-Methylnicotinamide	M+Na	160.0607

HILIC/ +	425.2159	1E-02	0.9	↑ Combo X Control; CMO	1b,3a,7b-Trihydroxy-5b-cholanoic acid	M+H	425.2897
HILIC/ +	456.2266	3E-02	0.9	↓ CMO X Control; Combo	Lithocholic acid glycine conjugate	M+Na	456.30843
Folic acid							
HILIC/ -	477.7417[#]	7E-09	1.7E-06	↑ CMO X Control; Combo	Pentaglutamyl folate	M-2H	477.6478
Neurotransmitter							
HILIC/ +	161.0923	6E-03	0.8	↑ CMO X Control; Combo	Tryptamine	M+H	161.1073
HILIC/ +	285.9893	2E-02	0.9	↑ CMO X Combo	Epinephrine sulfate	M+Na	286.0355
Steroid							
HILIC/ +	414.1791[#]	2E-02	0.9	↑ CMO X Control; Combo	Pregnenolone sulfate	$M+NH_4$	414.2309

↑ CMO X Control; Combo - increased presence of adduct in CMO treatment compared to control and combo treatments.

↑ CMO X Combo – increased presence of adduct in CMO treatment compared to combo treatment.

↑ Combo X Control – increased presence of adduct in combo treatment compared to control treatment.

↑ Combo X Control; CMO – increased presence of adduct in combo treatment compared to control and CMO treatments.

↓ CMO X Control; Combo - decreased presence of adduct in CMO treatments compared to control and combo treatments.

* Metabolite markers based on the VIP scores of PLS-DA.

[#] Similar metabolite found in the plasma of pup at weaning.

[$] Similar metabolite found in pup after 30 days of weaning.

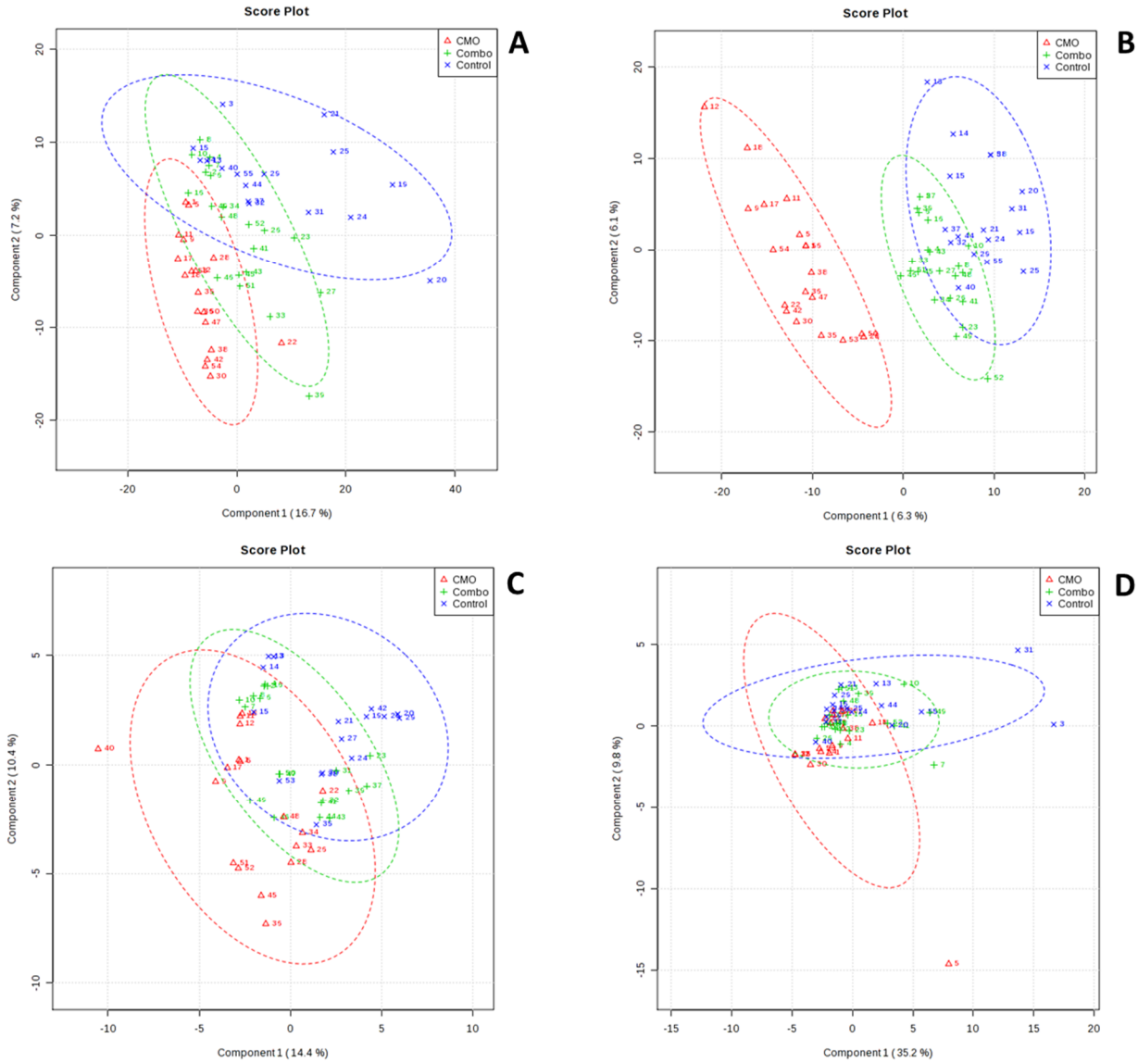

Figure 5.15 The partial least square analysis discriminant analysis (PLS-DA) cross-validated score plot generated from mass spectrometry spectra of plasma samples from pups at weaning from dams treated with control (+), combo (x) and caprine milk oligosaccharides (Δ) diet. Samples were run in two different columns HILIC and C18 and in both positive and negative ionisation modes separately. (A) HILIC +ve; (B) HILIC -ve; (C) C18 +ve; (D) C18 –ve.

Table 5.20 Summary of plasma metabolites from liquid chromatography-mass spectrometry analysis, HILIC and C18 columns, negative and positive mode, found to separate pups at weaning according to dams treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
Fatty acids and/ or mitochondrial derivatives							
HILIC/ -	377.6035	2E-24	1.8E-21	↑ CMO X Control; Combo	Trihexosylceramide (d18:1/24:0)	M-3H	377.5913
HILIC/ -	373.6079[&]	3E-24	1.8E-21	↑ CMO X Control; Combo	Phosphatidylcholines		
					Phosphatidylethanolamines		
HILIC/ -	375.6058	3E-23	1.3E-20	↑ CMO X Control; Combo	Phosphatidylserines		
HILIC/ -	379.6013	4E-23	1.4E-20*	↑ CMO X Control; Combo	Phosphatidylcholines		
					Phosphatidylethanolamines		
					Phosphatidylserines		
HILIC/ -	331.6558	8E-23	2.3E-20	↑ CMO X Control; Combo	Ceramides		
HILIC/ -	371.6101	2E-22	4.3E-20	↑ CMO X Control; Combo	Tetracosanoyl-CoA	M-3H	371.4827
HILIC/ -	471.74808[&]	1E-21	1.3E-19	↑ CMO X Control; Combo	Lactosylceramide (d18:1/22:0)		
					cis,cis-3,6-Dodecadienoyl-CoA		
HILIC/ -	298.6857[&]	6E-21	8.1E-19*	↑ CMO X Control; Combo	Triglycerides		
HILIC/ -	260.9807	8E-20	9.0E-18	↑ CMO X Control; Combo	Triglycerides		
HILIC/ -	393.8184[&]	1E-19	1.1E-17	↑ CMO X Control; Combo	Phosphatidylcholine,		
					phosphatidylserine or		
					phosphatidylethanolamine		
					Clupanodonyl carnitine	M+Na	497.3397
					Lysophospholipid	M+Na	496.2435

C18/ +	274.2000	0.2	*	↑ Combo X CMO	Palmitic acid	M+NH_4	274.2741
C18/ -	234.1875	2E-02	0.6	↑ Combo X CMO	Cholesteryl pentanoate	M-2H	234.1989
C18/ -	453.2000	0.2	*	↑ Combo X CMO	Cyclic phosphatidic acid	M+Cl	453.2178
Bile acids or liver derivatives							
HILIC/ +	455.2050	9E-04	0.2	↑ CMO X Control; Combo	3 beta,7 alpha-Dihydroxy-5-cholestenoate	M+Na	455.3132
HILIC/ +	160.0606[&]	3E-02	0.7	↓ CMO X Control; Combo	1-Methylnicotinamide	M+Na	160.0607
C18/ +	424.3000[$]	0.05	*	↓ CMO X Control; Combo	7-Ketodeoxycholic acid	M+NH_4	424.3057
					3-Oxocholic acid	M+NH_4	424.3057
					3,7-Dihydroxy-12-oxocholanoic acid	M+NH_4	424.3057
Protein degradation and derivatives							
HILIC/ +	168.0774	4E-03	0.5	↑ CMO X Control; Combo	(S)-2-amino-6-oxohexanoate	M+Na	168.0631
					Allysine	M+Na	168.0631
HILIC/ +	226.1287	6E-03	0.5	↑ CMO X Control; Combo	Asparaginyl-Alanine	M+Na	
C18/ +	229.2000	9E-03	0.6	↑ CMO X Control; Combo	Isoleucyl-Proline or Leucyl-Proline	M+H	229.1547
HILIC/ +	204.0563	2E-02	0.7	↓ CMO X Control; Combo	L-Tyrosine	M+Na	204.0631
HILIC/ +	166.0867	2E-02	0.6	↓ CMO X Control; Combo	L-Phenylalanine	M+H	166.0863
HILIC/ +	160.0606[&]	3E-02	0.7	↓ CMO X Control; Combo	3-Dehydrocarnitine	M+H	160.0968
HILIC/ +	245.075[&]	4E-03	0.5	↓ CMO X Control; Combo	L-Glutamic acid 5-phosphate	M+NH_4	245.0533
					Uridine	M+H	245.0768
HILIC/ +	126.0220	4E-03	0.5	↓ CMO; Combo X Control	Taurine	M+H	126.0219
Steroid							
HILIC/ +	414.1791[&]	7E-04	0.2	↑ CMO X Control; Combo	Pregnenolone sulfate	M+NH_4	414.2309

Thyroid hormone derivatives							
HILIC/ +	421.9221	2E-03	0.5	↓ CMO; Combo X Control	Monoiodothyronine	M+Na	421.9860
Folic acid							
HILIC/ -	477.7417[&]	6E-22	9.1E-20	↑ CMO X Control; Combo	Pentaglutamyl folate	M-2H	477.6478
Vitamin D							
C18/ -	431.2718[$]	0.2	*	↑ Combo X CMO	23S,25,26-Trihydroxyvitamin D3	M-H	431.3167
					Ergocalciferol	M+Cl	431.3086

↓ or ↑ CMO X Control; Combo – decreased or increased presence of adduct in CMO treatment compared to control and combo treatments.

↑ Combo X CMO – increased presence of adduct in combo treatment compared to CMO treatment.

↓ CMO X Control; Combo - decreased presence of adduct in CMO treatments compared to control and combo treatments.

* Metabolite markers based on the VIP scores of PLS-DA.

[#] Similar metabolite found in the plasma of pup at weaning.

[$] Similar metabolite found in pup after 30 days of weaning.

components from the mitochondria and/or related to fatty acid metabolism such as N-heptanoyglycine, 3-dehydrocarnitine, cic,cic-3-6-dodecadienoyl-CoA, cardiolopins, glutaryl-, CoA, triglycerides and (S)-Hydroxyhexanoyl-CoA. Neurotransmitters, such as tryptamine and epinephrine sulphate; and metabolites involved in steroid metabolism, such as pregnenolone sulphate, were in higher concentrations in dams fed the CMO diet compared to dams fed the control and combo diets. Other important putative metabolites like ceramide pentaglutamyl folate (folic acid), 1 methylnicotinamide, phosphatidilcholines and phosphatidylethanolamides were also in higher concentrations in CMO-fed dams. Combo-fed dams had an increased concentration of plasma bile acids such as 1b,3a,7b-trihydroxy-5b-cholanoic acid when compared to control and CMO-fed dams, and this was in agreement with those same metabolites found in urine.

PLS-DA plots of plasma metabolites from pups at weaning (Figure 5.15) showed a clear clustering with diet and clear separation between combo and CMO clusters in HILIC negative plot. A summary of discriminating metabolites in pups that can be ascribed to the diet of the dam are described in Table 5.20. Full metabolite descriptions are presented in Appendix G. At weaning, pups from CMO and combo-fed dams had increased concentrations of metabolites associated with fatty acid metabolism, such as ceramides, triglycerides, tetracosanoyl-CoA, cic,cic-3-6-dodecadienoyl-CoA, clupanodonyl carnitine, lysophospholipids, palmitic acid, cholesteryl pentanoate and cyclic phosphatidic acid, some of which were also found in dams fed the CMO diet. At weaning, there were also a range of changes in the metabolites from bile acids (e.g. 3 beta, 7 alpha-Dihydroxy-5-cholestenoato, 7-Ketodeoxycholic acid, 3-Oxocholic acid, 3 beta,7 alpha-Dihydroxy-5-cholestenoate and 3,7-Dihydroxy-12-oxocholanoic acid) in pups at weaning from CMO-fed dams. CMO-fed dams also had a range of changes in the level of metabolites involved in protein degradation (Allysine, Asparaginyl-Alanine, and other amino acids). At weaning, different concentrations of other important metabolites, such as, pregnolone sulphate monoiodothyronine and pentaglutamyl folate (folic acid), were observed in pups from dams fed the CMO diet. At

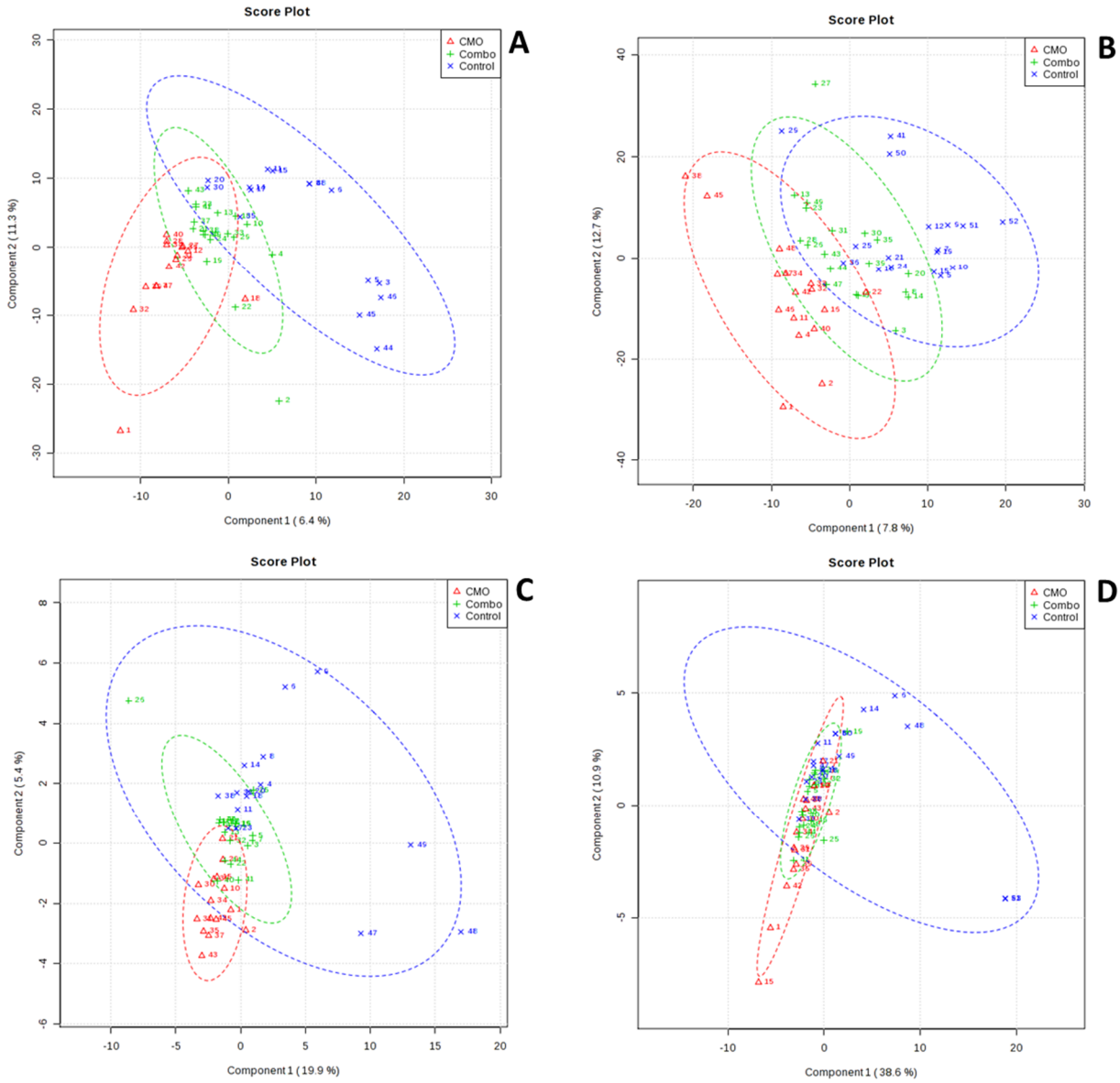

Figure 5.16 The partial least square analysis discriminant analysis (PLS-DA) cross-validated score plot generated from mass spectrometry spectra of plasma samples from pup 30 days after weaning from dams treated with control (+), combo (x) and caprine milk oligosaccharides (Δ) diet. Samples were run in two different columns HILIC and C18 and in both positive and negative ionisation modes separately. (A) HILIC +ve; (B) HILIC -ve; (C) C18 +ve; (D) C18 –ve.

Table 5.21 Summary of plasma metabolites from liquid chromatography-mass spectrometry analysis, HILIC and C18 columns, negative and positive mode, found to separate pup 30 days after weaning according to dams treatment.

Column/Mode	M_{LCMS} [Da]	p.value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
Fatty acids and/ or mitochondrial derivatives							
HILIC/ +	120.0636	0.01	0.8*	↑ CMO X Control; Combo	Acetoacetic acid	$M+NH_4$	120.0655
					L-Threonine	M+H	120.0655
HILIC/ +	195.9779	0.04	0.8*	↑ CMO X Control; Combo	3-Hydroxyhippuric acid	M+H	196.0604
HILIC/ -	480.3111	0.03	0.6*	↑ CMO X Combo	Lysophospholipid (18:0/0:0) or LysoPC(15:0)	M-H	480.3096
C18/ +	544.3000[#&]	0.01	0.2*	↑ CMO X Control; Combo	Lysophospholipids	M+Na	544.3374
C18/ +	374.3000	0.01	*	↑ CMO X Control; Combo	Tetracosahexaenoic acid	$M+NH_4$	374.3054
					13,14-Dihydro PGF2a	$M+NH_4$	374.2901
					Prostaglandin F1a	$M+NH_4$	374.2901
					Monoacylglyceride (18:1(9Z)/0:0/0:0)	$M+NH_4$	374.3265
					14-HDoHE	$M+NH_4$	374.2690
					Sphingosine 1-phosphate (d16:1-P)	M+Na	374.2067
C18/ +	520.3000			↑ CMO X Control; Combo	Lysophospholipid (18:2(9Z,12Z))	M+H	520.3398
Bile acids or liver derivatives							
C18/ +	424.3000[#]	0.03	0.2*	↓ Combo X Control	7-Ketodeoxycholic acid	$M+NH_4$	424.3057
					3-Oxocholic acid	$M+NH_4$	424.3057
					3,7-Dihydroxy-12-oxocholanoic acid	$M+NH_4$	424.3057
Protein degradation and derivatives							

HILIC/ +	355.1739	0.01	0.8	↑ Combo X Control; CMO	Lysyl-Tryptophan or Tryptophyl-Lysine	M+Na	355.1741
HILIC/ -	167.0204	0.004	0.6	↑ Combo X Control; CMO	Glutaric acid	M+Cl	167.0117
					Uric acid	M-H	167.0211
HILIC/ -	88.0360	0.005	0.6*	↓ Combo X Control; CMO	Sarcosine	M-H	88.0404
					L-Alanine	M-H	88.0404
HILIC/ -	323.0392	0.009	0.6*	↑ Combo X Control; CMO	Orotidine	M+Cl	323.0288
					Uridine 5'-monophosphate	M-H	323.0286
Vitamin D							
C18/ -	431.2718[#]	0.005	0.1*	↓ CMO X Control; Combo	23S,25,26-Trihydroxyvitamin D3	M-H	431.3167
					Ergocalciferol	M+Cl	431.3086

* Metabolite markers based on the VIP scores of PLS-DA.

[#] Similar metabolite found in the plasma of pup at weaning.

[&] Similar metabolite found in the plasma of dams

weaning, pups from dams fed the combo diet had increased concentrations of plasma trihydroxyvitamin D3 or ergocalciferol (vitamin D2). In contrast to plasma from pups at weaning, PLS-DA plots from pups 30 days after weaning (Figure 5.16) showed a less clear separation between the treatment groups. The summary of important metabolites in pups 30 days after weaning is described in Table 5.21. Full metabolite descriptions are presented in Appendix H. 30 days after weaning, pups from dams fed the combo diet had increased concentrations of metabolites related to protein degradation (Lysyl-Tryptophan, glutaric acid, orotidine) and decreased bile acid metabolites (Ketodeoxycholic acid or Oxocholic acid). 30 days after weaning, pups from CMO-fed dams had an increase in putative metabolites involved in fatty acid metabolism (acetoacetic acid, threonine, 3-Hydroxyhippuric acid, lysophospholipids, monoacylgliceride and 14-HDoHE) and decreased vitamin D (Trihydroxyvitamin D3 or ergocalciferol) metabolites when compared to pups from the control and combo groups.

5.8 Discussion

This study investigated the effects of CMO supplemented to conventionally raised dams on the maternal large intestine microbiota on the development of the offspring. Consumption of CMO by the dams, during gestation and lactation was associated with increased maternal colon length and altered caecal SCFA production with increasing formic acid and decreasing isobutyric concentrations. Dietary CMO, however, had no effects on dams colon microbiota and morphology. These results disprove the first hypothesis, which stated that CMO consumption by the dams would promote a bifidobacteria-enriched microbiota leading to an improved bacterial fermentation and morphology in the large intestine. Increased milk protein concentration was associated with increased pups body weight and length and increased proportions of bifidobacteria in the colon of pups at weaning, which confirmed the second hypothesis. In addition, changes in the dams, fed CMO diet, plasma and urine putative metabolites related to bile acid and fatty acid metabolism were also observed in the pups. 30

days after weaning, pups from CMO-fed dams had increased body fat compared to pups from control and combo-fed dams. There were no changes observed in the pups at weaning that could be detected 30 days after weaning, disproving the third hypothesis, which stated the effects of maternal CMO-supplementation on the offspring large growth and GIT microbiota composition are detected after 30 days consuming control diet.

5.8.1 Diet composition

The CMO and combo diets were thought to contain the same nutrient content, with the only difference being the presence of CMO, replacing equivalent amounts of dietary fibre and sugars in the combo diet. Thus, any variation in experimental data obtained from the groups could be attributed to the CMO content. However, the combo diet was shown to have double the amount of glucose and galactose, twenty four times more lactose and five times more GOS than the CMO diet. These variations may have occurred due to changes (from before and after diets being made) in the methodology used to quantify the concentration of these sugars in the CMOP, which was used to calculate the amount of lactose, glucose, galactose and GOS added to the combo diet. The concentration of GOS present in the combo diet, however, was similar to the concentration of CMO in the CMO diet at around 1%. Previous studies which tested the dietary effect of GOS or other prebiotic oligosaccharides, such as FOS or inulin, on the GIT function, were added at a rate of 1 to 5% [246, 248, 469-472]. Therefore, effects of the combo diet on the parameters measured for the dams and pups may be attributed to its GOS content [244, 247, 254, 473].

Control, combo and CMO diet were shown to contain vitamin D2 which is not present in the vitamin mix (V10001) normally added to the basal AIN-76Adiet. The diet supplier, Research Diets Inc, reported that vitamin D2 may have been added with the casein, the protein source of AIN-76Abased diets. In addition, higher concentration of vitamin D2 was found in combo and CMO diets compared to the control diet, and indicate that the GOS and CMOP added to the combo and the CMO diets respectively, may also have included this vitamin as an

additional component of the diets. Therefore, increased vitamin D2 concentration in combo and CMO diet could have affected dams response, in particular dams metabolism [474, 475] and mineral absorption [476-478].

5.8.2 Pregnancy and pups development

Compared to the control diet, the CMO and combo diets [247] did not alter food intake, weight gain during pregnancy [247, 479], nor the number of offspring per dam [247, 473, 480]. In numerous studies on adult rodent models, prebiotic intake has been associated with a decrease in body weight and fat mass due to a reduction on food/energy intake and increased satiety [481-483]. Few studies have explored the effects of prebiotic diets during gestation and lactation on the mother/infant pair [27, 244, 247, 473, 479, 480, 484, 485]. Reduction of dam and offspring weight gain was only observed in pregnant mice fed 20% FOS [479, 484, 485]. Maurer et al. [473] did not observe any difference in body weight gain between the offspring of dams fed a high-fibre diet (mix of oligofructose–inulin with a 1:1 w/w), a high-protein diet or a control diet.

CMO and combo diet had a significant impact on offspring body weight [247] and length at weaning. The pups of the dams fed the CMO diet were longer and heavier at weaning. These differences in pups body weight and length may be associated with an increase in colon length [247], liver weight [473] and increased muscle mass, and no change in fat mass. Unaltered leptin levels and visceral fat weight support the former. Conversely, 30 days after weaning, pups from dams fed the CMO diet had an increase in leptin concentration and fat mass without change in body weight. In agreement with this data, Desbuards *et al*, [486] reported a trend for increased leptin concentration in pups 28 days after weaning which had consumed the same prebiotic diet as the dams.

5.8.3 Milk protein and lipid metabolism

The present study is the first to identify changes in milk protein concentration and maternal/pups lipid metabolism by maternal oligosaccharides consumption. Dams fed the CMO diet had increased protein content in milk, which may had contributed to increasing body weight and length of the pups at weaning. The concentration of protein reported in murine milk, however, is highly variable (32 and 125 mg/mL) [487, 488]. Therefore, it is uncertain whether the protein concentration in the milk of the dams fed the CMO and combo diets would cause any physiological effect in the pups. The CMO diet decreased fat concentration in milk compared to the control diet, however, due to the small number of samples analysed (n = control, 1; combo, 3; CMO, 2); these data should be interpreted with caution.

Early nutrition affects expression of genetic growth potential; this can have short-term and long-term effects on growth, development, metabolic programming, and disease risk [489]. In neonatal rats, higher protein intake via the enteral route was reported to enhance short term weight gain, insulin resistance, and modified expression of glucose transporters. However, these differences were not sustained in the adult life [490]. In human neonates, high protein milk formula (2 vs. 1.5 g/100 mL) fed to babies considered small for gestational weight [491] showed, an increase in total adiposity between 2 and 6 years of age [492, 493].

The levels of oligosaccharides in milk from dams consuming CMO was not determined in this study due to the small sample volumes obtained. Any potential oligosaccharide variations in milk composition could be associated with the differential colon growth in pups at weaning. At weaning, the increases in the abundance of *Bifidobacterium* spp. (control, 0.05%; combo, 0.07%; CMO, 1.37%) in the colon of pups from the dams fed CMO may be due to changes in the milk oligosaccharides profile of the dam favouring these commensal bacteria [494]. Hallam, *et al* [494], for example, showed that increasing protein or fibre content in the maternal diet during pregnancy and lactation modified milk oligosaccharides

content and GIT microbiota of dams which increased the numbers of bifidobacteria in the pups GIT microbiota. It is also plausible that limited direct exposure to the diets may have occurred when pups began to eat the diet of the dams as they approached weaning [495].

Despite no milk variations in lipid concentration in the dams fed the control, CMO and combo diets, there was a decreased liver weight and an increased bile acids, fatty acids and metabolites involved in steroid metabolism found in the plasma and/or urine of dams fed CMO and combo diets. This may be an indication that maternal lipid metabolism was altered in CMO and combo fed dams. Decreased liver weight was also reported in obese mice treated with a 1:1 mix of inulin and oligofructose, likely due to the lower fat accumulation in the liver [496]. Increased levels of bile acids and metabolites involved in fatty acid metabolism and increased liver weight were also found in pups from dams fed combo and CMO diets. Interestingly, Maurer et al [473] reported a trend to lower liver weight in weaned pups from dams fed high fibre diet.

It is unclear whether the CMO and combo diets affected dietary fat absorption through liver and bile salts metabolism or through modifications in the GIT microbiota. Interactions between GIT microbiota and bile salts are well known [497]. The bacterial metabolism of bile salts in the GIT lumen can involve deconjugation and dehydroxylation, converting primary bile salts such as cholate and chenodeoxycholate into secondary bile salts (e.g., deoxycholate and lithocholate). Bacterial metabolism of bile salts is partly responsible for the portion of the pool that is newly synthesised per day. Bacterial metabolism may also influence the physiological activity of bile salts since secondary bile salts are more hydrophobic than primary bile salts and therefore have a greater capacity to interact with dietary fat [497].

5.8.4 Gastrointestinal microbiota modulation by diet

No significant changes in relative abundance of bifidobacteria and/or lactobacillus were found in dams fed either combo or CMO diet. This is in disagreement with studies which have shown that the consumption of prebiotics by adults (human and rodents) and also pregnant mice changes the GIT microbiota composition, especially increasing the faecal concentrations of bifidobacteria and/or lactobacillus [27, 244, 245, 498]. Members of Firmicutes and Bacterioidetes, more specifically, genera *Oscillibacter* spp. (class Clostridia) and *Odoribacter* spp. (class Bacteroidia), respectively, were enriched in the colon of dams fed CMO diet compared to that of dams fed combo and control diets. Recent data suggest that the abundance of *Oscillibacter* spp [499, 500] and *Odoribacter* spp [501] are increased in obese individuals and negatively correlated with a functional intestinal barrier. Although, no difference was found in dams body weight or visceral fat, a range of changes in the metabolites from bile acids and fatty acids metabolism were found.

The effects of diet in the GIT microorganisms are important factors controlling energy balance and nutrient absorption, such as protein, carbohydrates and lipids. Semova *et al* [502] recently proposed four non-exclusive mechanisms by which the microbiota might stimulate fatty acids absorption and lipid droplet accumulation in enterocytes. First, the microbes might increase the bioavailability of fatty acids by modifying the production or composition of bile salts. Secondly, the microbes could directly contribute to luminal lipolytic activity that increases the availability of the fatty acids for absorption by the GIT epithelium. Thirdly, the microbes might enhance fatty acids absorption indirectly by evoking physiologic responses in the epithelium that stimulate its inherent absorptive capacity. Finally, the microbiota might reduce rates of fatty acid oxidation in epithelial cells resulting increased storage of fatty acids in lipid droplets.

In agreement with the limited data in this field [244], the specific changes observed in the microbiota of CMO-fed dam (increased proportions of *Oscillibacter* spp. (class Clostridia)

and *Odoribacter* spp. (class Bacteroidia)), were not observed in the microbiota of the pups at weaning. Weaned pups from dams fed CMO diet, however, had an increased relative abundance of bifidobacteria. No identification to species level was possible through the analysis of the 454 NGS data generated in this study; therefore, the significance of this altered bifidobacterial relative abundance was not able to be determined. The role of bifidobacteria on the host energy metabolism is strain dependent; different strains have been shown to differently affect host body weight, liver weight and fat distribution [503, 504]. *B. longum*, for example, exhibited a strong effect in lowering serum total cholesterol both in rats and humans [505]. In contrast, a combination of *Streptococcus thermophilus* and several species of lactobacillus and bifidobacteria was found to increase liver fat with no metabolic or body weight changes in a clinical trial [506].

The increase in the proportions of bifidobacteria strains observed in pups at weaning from CMO-fed dams was not observed in pups 30 days after weaning. Species of the genus *Barnesiella* spp., *Parabacteroides* spp. and *Allobaculum* spp., were shown to be abundant in this group. Proportions of *Barnesiella* and *Parabacteroides* genera and members of the Clostridia class were also shown to be increased in the colon of pups from dams fed the combo diet, 30 days post-weaning. Little is known about the effects of the *Barnesiella* and *Parabacteroides* genera on the host physiology. *Allobaculum* spp., however, were reported to be dominant in the intestine of rats and hamsters fed with algal dietary fibers [507], and supplemented with grain sorghum lipid extract [508], respectively, which was correlated with cholesterol metabolic improvement. Ravussin, *et al* [509] also reported that low-fat feeding in mice was associated with an increase in the genus *Allobaculum* compared with high fat feeding. Increased members of the class clostridia have been associated with obesity [510, 511] which is in agreement with the high visceral fat content found in these pups 30 days after weaning.

The CMO diet impacted caecal microbial fermentation through an increase in the concentration of formic acid and a decrease in the concentration of isobutyric acid, compared to the control diet. Formic acid is a volatile SCFA rarely detected in nutritional studies [512]. This might be attributed to the fact that formic acid is likely formed by microorganisms in the colon only at the initial phase of substrate fermentation. Furthermore, formic acid is an intermediate product, not an end-product, of bacterial fermentation and is converted readily to CO_2 and water [513]. In a study comparing the SCFA of healthy subjects with inflammatory bowel disease patients, faecal formic acid, for example, was only detected in healthy subjects [512]. Other common end-product of bacterial fermentation, isobutyric acid is produced by fermentation of proteins [514]. Fermentation of protein in the large intestine increases as carbohydrate availability becomes a limiting factor for microbial fermentation [515]. Collectively, this could explain the decline in isobutyric acid in the caecum of CMO and combo-fed dams, as the preferential metabolism of CMOP and GOS by carbohydrate-fermenting bacteria may have altered the microbiota and associated fermentation towards a type that was less proteolytic [516].

At weaning, pups from dams fed CMO had increased concentration of butyric acid compared to pups from fams fed combo and control diets. Of the major SCFAs, butyrate is of special interest because it is the preferred energy source for colonocytes [517] and promotes a normal phenotype in these cells and hence may protect against cancer and other serious colonic diseases [518]. Increased consumption of dietary fibres and/or production butyric acid [519] were also associated with increased intestinal length [520] which is in agreement with the increase on dams and pups colon length at weaning observed. However, the effects on colon length are usually accompanied of increase in weight and crypt size [521-523] which was not observed in the present study.

Combo-fed dams and their pups, 30 days after weaning, showed an increase in SCFA propionic and butyric acid concentration, typically produced by carbohydrate fermentation.

These results are in agreement with previous studies demonstration that the end-products of GOS fermentation by murine caecal microbiota were propionate and butyrate [451]. It has been demonstrated that propionic acid lowers fatty acids content in liver [524] and plasma [525], reduces food intake [526], exerts immunosuppressive actions [527] and may improve tissue insulin sensitivity [528, 529].

5.8.5 Calcium, magnesium and zinc concentrations in the femur

In the present study the CMO diet had no effect on the dams and pups femur mineral composition. The combo diet, however, increased zinc concentration in the femur of dams and increased zinc, calcium and magnesium concentrations in the femur of their weaned pups. Several studies in animals and humans have shown positive effects of nondigestible oligosaccharides on mineral absorption and metabolism, bone composition and architecture. These include inulin [530], oligofructose [531], FOS [532] and GOS [533-536]. GOS supplementation was shown to have positive effects on calcium and magnesium absorption. An increased concentration of zinc in mice femur, however, was only observed by the combined intake of dairy product fermented by lactobacilli and GOS at 5% inclusion in the diet [537].

Mineral composition in dams and pups femur may also have been influenced by the higher vitamin D levels found in the combo and CMO diets compared to control diet. Non-active dietary vitamin D is converted by 25-hydroxylase in the liver to 25-hydroxyvitamin D [25(OH)D], which is then activated by 1a-hydroxylase in the kidneys to become 1,25-dihydroxyvitamin D [1,25(OH)2D]. The active vitamin D, acts in the intestine to improve calcium, zinc and magnesium absorption and maintain mineral balance [538, 539].

5.8.6 Metabolites

The PLS-DA showed that CMO and combo diet affected urine and plasma metabolites in dams and weaned pups. These effects may have been caused by modulation of the GIT

microbiota (prebiotic effect) and/or by the ingested oligosaccharides, which potentially could reach the plasma and directly modulate cell signalling [540]. Recently, ingested HMO were found in the blood circulation and urine of breast-fed infants with levels corresponding to mother's milk [540]. HMOs were shown to be transported across the GIT epithelium by receptor-mediated transcytosis as well as via paracellular pathways [541]. Milk oligosaccharides have also been hypothesised to be taken up from the GIT lumen by dendritic cells [542]. Moreover, FOS, which are carbohydrates added to infant formula to mimic HMO, could also be detected in the urine of formula-fed infants [543].

The alteration of the concentrations of bile acids and fatty acid metabolites, in both urine and plasma, together with the increase in liver weight in dams fed CMO and combo diet, suggest alterations in lipid metabolism. The same effects were found in plasma of pups at weaning and in plasma and urine of pups 30 days post-weaning. Lipid metabolism involves the digestion of dietary lipids to free fatty acids by lipases within the lumen [544] which are absorbed by enterocytes by bile salts solubilisation [545]. Fatty acids are then oxidised to generate energy or stored in tissues such as liver [546]. Hepatic gene expression was shown to be regulated by prebiotic intake in an obese rat model [496] and also in the offspring of dams fed a high fibre diet [473]. It is difficult, however, to reconcile the effects of prebiotics on the bile salts and hepatic metabolism in the dams with the consequences for bile salts and hepatic metabolism in their pups in the present study.

Dams had an increase in the concentration of sex steroid hormones in their urine when fed combo (estrone, epiandrosterone, ethiocolanolone, hydroxyprogesterone) and CMO diets (hydroxyestradiol, epiestriol). CMO and combo diets may have directly affect the sex hormones synthesis, by acting on the gonads, the adrenal gland, and the placenta metabolism [547], or by indirect affecting the metabolism or composition of the GIT microbiota [547, 548]. Bacteria are capable of metabolising sex steroid hormones through the activity of distinct enzymes such as hydroxysteroid dehydrogenase that regulate the

balance between active and inactive steroids [547]. The dominating phyla that were identified to express these enzymes were Actinobacteria, Proteobacteria, and Firmicutes, largely represented in the GIT microbiota [548]. The effects on sex steroids were also observed in pups 30 days after weaning from combo-fed dams, which indicate that the effects may only be seem after sexual maturation.

1-Methylnicotinamide was increased in the urine of dams fed combo and CMO diets, and decreased in the plasma of pups from CMO-fed dams, at weaning. 1-Methylnicotinamide is major metabolite of nicotinamide metabolism (vitamin B3, niacin) and has been considered as a biologically inactive or even toxic molecule [549, 550]. An increase in urinary N-methylnicotinamide, for example, has been reported as a marker for cirrhosis [550] Recently, anti-inflammatory efficacy of 1-Methylnicotinamide after its topical application was demonstrated in patients with skin diseases [551, 552]. In addition, during *in vivo* studies, it was discovered that 1-Methylnicotinamide displays anti-thrombotic, anti-inflammatory, anti-diabetic and gastro-protective activities [553-556], as well as lowering triglycerides [557].

Another important metabolite related to vitamin B, folic acid (vitamin B9), was also increased in dams fed the CMO diet and their pups at weaning. Vitamin B9 is essential for numerous bodily functions. Humans cannot synthesise folates *de novo*; therefore, folic acid has to be supplied through the diet to meet daily requirements. Folic acid is also synthesised by some members of the GIT microbiota, inclusing *B. bifidum*, *B. infantis*, *B. breve*, *B. longum*, *Enterococcus faecalis*, *E. coli* and some streptococci [558]. Therefore, CMO may have stimulate members of the GIT to synthesise folate to the benefit of dams and pups. The human body needs folate to synthesise DNA, repair DNA, and methylate DNA as well as to act as a cofactor in certain biological reactions [559]. It is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy [560].

To date, most studies have utilised an untargeted approach to obtain a comprehensive profile of the altered metabolites. While this approach has discovery potential, it also has

drawbacks. Simultaneous quantification of a large number of metabolites using MS remains challenging due to the large dynamic range of metabolites (up to 9 orders of magnitude [561]) and sensitivity limits. Without a predetermined set of targets, extraction protocols and MS operating parameters cannot be tailored to improve sensitivity. Unambiguous identification of bacterial metabolites could also present challenges if the sample contains many ion fragments that have the same mass signatures.

5.8.7 Conclusions

In conclusion, consumption of CMO by the dams, during gestation and lactation had no effects on maternal GIT microbiota and morphology which is in agreement with the data from Chapter 4. Changes in lipid metabolism and milk protein concentrations of the dams were observed. These modifications may have improved the development of the pups, the relative abundance of bifidobacteria and production of butyric acid in the colon, at weaning. Changes in the plasma and urine metabolites involved on bile acids and fatty acids metabolism were also observed in the pups as a consequence of their dams consuming the CMO diet.

The effects of maternal CMO diet on pups consuming control diet for 30 days after weaning, were mostly washed out; however, an increased body fat was observed. The significance of this finding is unknown, but it is in agreement with recognised detrimental effects caused by a mismatch between the environments encountered during development and adulthood. In contrast to the CMO diet, the consumption of combo diet by the dams increased butyrate production and the femur mineral composition in dams and weaned pups. The combo diet had also similar effects to CMO fed dams on maternal lipid metabolism, milk protein composition and consequently on pups development. These findings indicate that, to some extent CMO may have the same beneficial effects of GOS, prebiotic oligosaccharides with well described health effects. Further studies, however, are needed to deeply understand the physiological effects of CMO in the maternal/infant pair.

Chapter 6 General Discussion

Chapter 6

6.1 Background

The “Developmental Origins of Health and Disease” hypothesis established the principle that the incidence of non-communicable adult diseases may be linked to *in utero* development. As described in Chapter 1 maternal diet, during pregnancy and lactation, was identified as one of the most influential, but modifiable, factors that promotes altered foetal growth and organ development. The effects of maternal GIT microbiota on the development and maturation of the neonatal GIT have also been reported [228, 399, 400]. The maternal GIT microbiota was shown to be transferred to the foetus/neonate through the placenta, during delivery and breastfeeding. The importance of the neonatal GIT development and maturation to the proper function of immune system and incidence of metabolic diseases have also been reviewed in Chapter 1.

Few studies have explored the effects of prebiotic diet administered during gestation on the development of the offspring [27, 244, 247, 473, 480, 484, 485]. Fujiwara et al. [27, 246], for example, demonstrated that dietary supplementation of dams with FOS changed the GIT microbiota profile of the offspring and diminished the severity of atopic dermatitis. In contrast, the combined dietary supplementation of GOS and FOS increased the proportions of bifidobacteria in the maternal GIT, but the effect was not transferred to the offspring [244]. Another study showed that a GOS and inulin-enriched diet fed throughout dams pregnancy and lactation was able to increase offspring colon length and body lean weight [247].

HMO are oligosaccharide prebiotics naturally present in human milk, known to improve the infant GIT colonisation and the immune response [446, 447]. There has been interest in sources of complex oligosaccharides similar to those within human milk, which could be used as functional ingredients for human health. Natural prebiotic oligosaccharides, similar to HMO, have been found in the milk of all mammals studied so far [145, 301, 449]. Among

domestic farm animals, caprine milk has oligosaccharides structurally similar to human milk [265] and potentially similar beneficial effects on the infant [450]. Although a few milk oligosaccharide enrichment methods have been developed, utilising bovine whey to support the utilisation of these oligosaccharides in *in vitro* and *in vivo* studies, this methodology still needs to be applied and tested in other milk types, such as caprine milk.

The hypotheses of this thesis were that: 1, Bifidobacteria selected from the faeces of breast-fed infants are able to ferment CMOP and accelerate the development and maturation of the mono-associated mice large intestinal mucosa; 2, Dietary CMO directs the maternal large intestine microbiota toward one with increased fermentative capacity leading to increased milk nutritive value; and 3, Dietary CMO-induced changes in the maternal large intestine microbiota and milk composition, accelerates the development and maturation of the large intestine tissue of the pups and alters the GIT microbiota composition.

The main aims present in this dissertation were:

1. Purify CMO from liquid whey to obtain a CMOP (Chapter 2) required for the *in vitro* and *in vivo* studies described in Aims 2 to 5.

2. Obtain bifidobacteria from the faeces of breast-fed human infants and determine which are capable fermenting CMOP (Chapter 3) and test the effects of the best strain, determined by enhanced CMO fermentation, on the morphology of the colonic mucosa of GF supplemented with dietary CMO (Chapter 4).

3. Analyse changes in the maternal metabolism, large intestine microbiota and fermentative capacity in response to dietary CMOP (Chapter 5).

4. Assess changes milk nutrient composition of lactating mice and offspring development in response to CMO-induced changes in dam metabolism and large intestine microbiota (Chapter 5).

5. Assess the long term effects of maternal CMO-consumption on the development of the offspring after 30 days consuming control diet (Chapter 5).

6.2 Summary

This dissertation presents an enrichment method for New Zealand Saanen CMO and the characterisation of CMO profile and its health effects through *in vitro* and *in vivo* studies. The oligosaccharides profile of New Zealand Saanen caprine colostrum, milk and whey were determined in Chapter 2. New Zealand Saanen milk and whey were shown to contain a similar oligosaccharide profile identified in studies with other caprine breeds [265, 301, 317-320]. The present study, however, only identified and quantified seven of the most abundant oligosaccharide, although 40 different oligosaccharides structures have been identified in caprine milk in a recent study [301].

The study present in Chapter 2 was the first to report NZ Saanen caprine colostrum, milk and whey composition, which is composed by high concentrations of health promoting sialyloligosaccharides. The sialyloligosaccharides (*N*-glycolylneuraminyl-lactose (*m/z* 648), 3'-sialyl-lactose (*m/z* 632) and 6'-sialyl-lactose (*m/z* 632)) are highly prevalent oligosaccharides in New Zealand Saanen caprine colostrum, milk and whey, although there was compositional variation between individual samples (Table 2.6). Sialyl-oligosaccharides have important health effects. 3'-sialyl-lactose and 6'-sialyl-lactose oligosaccharides, for example, have been shown to increase the number of bifidobacteria in neonatal GIT [282] and promote, in adult mice, an GIT microbiota resistant to DSS-induced colitis [562]. There are indications that it may reduce the severity of influenza virus infection and ulcers caused by *Helicobacter pylori* [284]. Other functions include increased immunity in infants, development of cerebral function and enhanced proliferation of commensal enteric bacteria [72, 237, 285, 286]. These results suggest that caprine whey from Camembert cheese manufacture is a rich source of oligosaccharides and the *m/z* data suggested that there are some common oligosaccharides between New Zealand Saanen caprine and human milk.

Chapter 2 also described an a multi-step approach to process caprine whey containing 0.3% oligosaccharides and produce an CMOP with a final CMO content of 8% (w/w) and with an oligosaccharides profile similar to the initial caprine milk. Caprine whey was chosen as the starting material for further enrichment of CMO as most of the fat and protein had already been removed during cheese making simplifying further oligosaccharide enrichment. After initial centrifugation and ultrafiltration steps, used to decrease the fat and protein concentration of the whey, β-galactosidase was used to hydrolyse the lactose to avoid lactose binding to the porous graphitised carbon under the adsorption/desorption conditions used to bind and elute the oligosaccharides. The method recovered approximately 80% of the original oligosaccharides present in the starting material, cheese whey. However, due to high concentration of lactose in the ultrafiltration permeate and incomplete lactose hydrolysis (92%), the major contaminants of the enrichment process were lactose, glucose and galactose. Although the final concentration of CMO-enriched product varied in Chapter 3, 4 and 5, the aim to obtain a CMOP needed for the *in vitro* and *in vivo* studies was achieved.

Variations in the composition of the CMOP were described in Chapters 3, 4 and 5 of this dissertation. These differences are attributable to variations in whey batches, ageing of the porous graphitised carbon and method of CMO quantification which was modified during the course of the PhD work. The composition of the experimental diets utilised in Chapters 4 and 5 were reanalysed at the end of the study, utilising all standards and described in the respective Chapters. The *in vitro* study, described in Chapter 3, tested the CMOP with the highest level of purity.

In Chapter 3, the effects of CMOP on growth of *B. breve*, *B. longum* and *B. bifidum* strains isolated from four exclusively breast-fed infants were determined. The CMO-enriched compound, containing high concentrations of sialyloligosaccharides (46%), enhanced the growth of all selected bifidobacteria isolated from breast-fed infants. CMO may also have stimulated the utilisation of lactose, glucose, galactose and GOS present in the CMOP. The

contrasting oligosaccharides, lactose, glucose, galactose and GOS utilisation and/or depletion observed between strains of the same species were in accordance with the bifidobacterial inter-strain heterogeneity shown in the RAPD analyses (Figure 3.1) and reported in previous studies [330, 360, 361]. Therefore, in agreement with the literature, although more than 8% of the identified genes from bifidobacterial genomes are predicted to be involved in carbohydrate metabolism, the ability to metabolise certain complex milk oligosaccharides is species specific [354, 376].

Among the bifidobacterial species tested, *B. bifidum* (AGR2166) utilised CMOP most efficiently when compared to *B. breve* and *B. longum* subsp*. longum,* which may indicate that *in vivo*, this strain may benefit the host CMO consumption. *B. bifidum* (AGR2166) was shown to ferment the 3'- and 6'-sialyl-lactose present in CMOP, in agreement with its growth profiles, where these same oligosaccharide isomers supplied as a sole source of carbon were also shown to support bifidobacterial growth (Figure 3.2). The greater depletion of 3'- and 6'-sialyl-lactose from CMO by *B. bifidum* (AGR2166) was likely through cell-associated sialidase expression, after induction with the same oligosaccharides. These data are in agreement with previous work suggesting that the enzyme sialidase resides intracellularly or on the surface of this species [356].

Augmented microbial biomass associated with enhanced growth and CMOP fermentation, increased the production of microbial fermentation end products such as the SCFA, acetate and lactate. *In vivo*, acetate is mainly metabolised in human muscle, kidney, heart, brain and liver [245]. It may play role in lipogenesis and contribute to inhibition of cholesterol synthesis [367]. Lactate does not accumulate in the human GIT and may be transformed to acetate, butyrate and/or propionate by the GIT microbiota [368]. The production of lactate by *B. bifidum* (AGR2166) *in vivo* was shown in Chapter 4.

The work presented in Chapter 3, therefore, demonstrates that CMO was able to differentially stimulate the growth of bifidobacteria, commonly found in the GIT of breast-fed

infants, and increase their fermentative capacity. *B. bifidum* AGR2166, shown to be able to ferment the CMO 3'- and 6'-sialyl-lactose was then selected for the *in vivo* study presented in Chapter 4.

Chapter 4 aimed to characterise the effects *B. bifidum* AGR2166 and dietary CMO on dams GIT. CMO and *B. bifidum* AGR2166 effects on pregnancy and bacterial translocation from maternal GIT to organs and pregnancy membranes were also determined. *B. bifidum* AG2166, independent of diet, was able to modulate maternal GIT function by decreasing weight of the GIT and increasing lactic acid production in the colon. Although, CMO was shown to increase *B. bifidum* translocation (as defined by the presence of 16S rRNA gene) from the GIT to the maternal MLN, liver, plasma and placenta, CMO was only able to increase GIT weight and no further effects on the GIT function and morphology were observed.

Low pregnancy rates, an expected characteristic of GF model [338, 412], were a limitation of this study. Neither the presence of *B. bifidum* AGR2166 in the GIT of females and males, nor dietary CMO were sufficient to reverse the effects related to the absence of microbiota on fecundity. Mice inoculated with bifidobacteria, however, showed an increase in the number of foetuses and also increase in foetus weight. An increase in foetus weight was also observed in dams fed the combo diet. The high fibre content of CMO (six times higher than combo) may have contributed to caecal enlargement [425-429] and consequently low organ function [418] and small foetal weight. The supply of nutrients to the foetus is the major influence that regulates its growth. It depends on the maternal body composition and size, nutrient stores, diet during pregnancy, transport of nutrients to the placenta and transfer across it [417]. GIT microbiota plays an important role in the regulation of energy balance and weight in animals. GF mice require 30% more calories than conventionally raised mice in order to maintain the same body weight [412] and therefore this factor may influence the level of energy available for the foetal growth. *B. bifidum* AGR2166 may have been able to

ferment the lactose present in the combo diet and increase the utilisation of this dietary compound to the benefit of the dams. The ability of AGR2166 to ferment lactose is shown in Chapter 3.

Although, there was no evidence of culturable bifidobacteria in the maternal extraintestinal tissues, bacterial translocation was inferred in all pregnant mice through the presence of the bifidobacteria 16S rRNA gene. Dams fed CMO diet showed an increase bacterial translocation compared to dams fed the combo diet. Dietary variables, such as dietary fibres, have been reported to increase bacterial translocation by increasing bacterial numbers in the large intestine [440]. Neither GF mice inoculated with *B. bifidum* AGR2166 nor conventionally raised dams fed CMO diet during gestation and lactation (described in Chapter 5), however, showed an increase in the relative abundance of bifidobacteria in the colon. In adult complex microbiota, longer feeding periods and/or high levels of dietary CMO may be required to observe changes in the GIT microbial composition [246].

The first goal of Chapter 5 was to characterise the effects of dietary CMO on dam metabolism, GIT and microbiota. Although dams fed CMO had increased colon length compared to dams fed the combo and control diets, no alterations in GIT microbiota and SCFA production were observed. Porphyromonadaceae, the only bacterial family with decreased proportions in the colon of dams fed CMO (Table 5.13), have been positively correlated with hepatic steatosis [563] and negatively correlated with intestinal concentrations of bile acids [564]. In agreement with the literature, dams fed CMO diet also showed decreased liver weight and increased modulation of urine and plasma putative metabolites involved in bile acids and fatty acids metabolism. Prebiotic [565, 566] and vitamin D intake, increased in both CMO and combo diet [474, 567], have also been reported to influence lipid metabolism [568]. Obese mice treated with a mix of oligofructose and inulin (1:1), for example, showed decreased liver weight, likely due to the lower fat accumulation in the liver [496].

The second goal of Chapter 5 was to assess changes in the milk nutrient composition of the dams and offspring development in response to CMO-induced changes in dam metabolism and large intestine microbiota. CMO-fed dams produced milk with increased protein concentrations when compared to control-fed dams, which may have impacted offspring growth by increasing body weight and length at weaning. Early nutrition affects the potential of genetic growth; this can have short-term and long-term effects on growth, development, metabolic programming, and disease risk [489]. In neonatal rats, for example, higher protein intake via the enteral route was reported to enhance short term weight gain, insulin resistance, and modified expression of glucose transporters. However, these differences were not sustained in adult life [490]. The increase in body weight of pups at weaning may also be associated with an increase in muscle mass, without increase in fat mass, as reflected by the unaltered leptin levels and visceral fat weight. Although no changes in visceral fat weight were observed in weaned pups at weaning from CMO fed dams, increased liver weight and increased levels of putative metabolites involved in the bile acids and fatty acid metabolism were found.

CMO-induced changes in dam metabolism were also observed in pups 30 days after weaning. 30 days after weaning, pups from dams fed CMO diet had increased plasma leptin concentration and fat mass without altering pup body weight. A trend to increased leptin concentrations in pups 28 days after weaning which had consumed the same prebiotic diet as the dams have been reported [486]. Maternal diet was also able to modulate urine and plasma putative metabolites involved in protein degradation, fatty acids and adrenal metabolism.

Combo diet, in Chapter 5, had higher concentrations of glucose, galactose, lactose and GOS compared to CMO diet. The concentration of GOS, in combo diet, was higher (around 1%) than CMO diet (around 0.2%). Previous studies which tested the dietary effect of GOS or other prebiotic oligosaccharides, such as FOS or inulin, on the GIT function, were added at a

rate of 1 to 5% [246, 248, 469-472]. GOS are among the most studied prebiotics. This prebiotic is fermented by the microbiota which generate SCFAs, such as acetate, butyrate, and propionate, that can modulate the expression of immunomodulatory genes [569]. The potential impact of GOS in the combo diet was observed in the dams, pups at weaning and pups 30 days after weaning. Dams fed the combo diet had increased production of butyrate and propionate in the caecum, increased protein in milk and increased zinc concentrations in the femur (Table 5.11). The combo diet increased the concentrations of putative metabolites involved in bile acids, fatty acids and adrenal metabolism (Table 5.17). The combo diet was also able to increase body length and zinc, calcium and magnesium concentrations in femur of the pups at weaning. Pups from dams fed combo diet had increased caecal propionate production and the proportions of members of the class clostridia in the colon microbiota, 30 days after weaning. Increased body fat weight and decreased urine metabolites involved in fatty acid metabolism were also observed. Several studies [570, 571] have shown that excessive weight gain during pregnancy increases the risk of developing hyperlipidaemia, insulin resistance and obesity in childhood. In the present study, the prebiotic diet affected neither bodyweight gain nor food of the dams intake during pregnancy. The increase in body fat found in pups 30 days after weaning from, both, dams fed combo and CMO diet may be explained by a mismatch between the environments encountered during development and postweaning, which may increase the risk of disease in later life.

6.3 Study limitations and future perspectives

Despite the obvious variations in the composition between BMO, CMO and HMO recently reviewed, the present study demonstrates that New Zealand Saanen caprine milk contains oligosaccharides (5 of 7 oligosaccharides identified) similar to human milk that are able to stimulate the growth of bifidobacteria. The high concentration of sialyloligosaccharides found in Saanen caprine milk in New Zealand provide a useful source for *in vitro* and *in vivo* experimentation of their modes-of-action and to further explore the potential health benefits.

A recent study has identified 40 different oligosaccharide structures in caprine milk and described the relative abundance of both acidic and neutral oligosaccharides using ultra-performance liquid chromatography [301]. The results of this current study use the relative intensities (peak areas) of extracted mass peaks from the mass spectrometric analysis for the quantification of CMO compared against the specific standards. While it is recognised that the intensity of each ion is depended on the ability of that particular molecule to ionise in solution, approximate amounts of each putative oligosaccharide were estimated based on the appropriate calibration curves. The seasonality of milk yield, caprine breed and degree of ionisation of the different oligosaccharides could be limitations of the current method being responsible for variations in CMO concentrations and detection when compared to other studies. Therefore, future studies are needed to confirm and compare the oligosaccharides concentrations of different caprine breeds, stage of lactations and diets. Also, more robust purification strategies, already developed to obtain high purity HMO and BMO enriched products should be applied to caprine milk to better understand their structural composition. Further knowledge on their structural composition will provide new insights into the complex biochemical pathways underlying their potential health promoting properties.

The CMOP obtained from New Zealand Saanen caprine milk was shown to be fermented by all bifidobacteria selected from breast-fed infants. Comparing the selected strains, *B. bifidum* isolates were able to better fermented CMO, especially the sialyloligosaccharides, which may indicate that *in vivo*, the colonisation and persistence this strain may be enhanced by the inclusion of dietary CMO. An absolute measurement of CMO utilisation by the bifidobacteria strains was impossible due to the high concentrations of lactose, GOS, glucose and galactose in the CMO enriched product. The manufacture of a CMOP with less contaminants is required for an improved understanding of oligosaccharide utilisation. Defining and linking the utilisation of specific oligosaccharide structures, such as the sialyloligosaccharides, to cultured bacteria will provide a scientific path for targeting infant

health by establishing protective microbial communities, beneficial to their hosts and potentially applicable to different stages of human life and health states.

The effects of CMOP and *B. bifidum* AGR2166 in the dams GIT and pregnancy outcomes were tested *in vivo*. Low levels of pregnancy were a limitation found in this study which should be considered when drawing any conclusions. CMO dietary effects on the dams GIT morphology, in both non-inoculated and inoculated mice, were not observed, however, the CMO effects on the bifidobacterial numbers were not analysed. Methods of microbial quantification such as Quantitative real time Polymerase Chain Reaction applied to the colon digesta of inoculated mice could have supported the inference of increased bacterial translocation observed in the CMO fed dams. The effects of CMO on other bacterial strains and bacterial translocation still need to be evaluated.

Using conventional mice, CMO were shown to modulate maternal metabolism, and increase milk protein concentration which may have affected the relative abundance and diversity of the offspring microbiota with developmental effects on the pups. A better understanding of the modifications observed in the milk through maternal diet is still required for any conclusions to be established. The maternal milk composition was not able to be fully elucidated in the present study due to the challenges to obtain enough milk samples. The effects of maternal CMO diet on other milk components such as the oligosaccharides, vitamins and specific fat and protein still remain to be evaluated. In general, there is lack of literature reagents on the influence of maternal prebiotic supplementation on milk nutritional composition and milk oligosaccharides composition, which may be associated with the changes found in the offspring GIT development.

The change between maternal supplementation and post-weaning diet may explain the detrimental effects, such as an increased pups body fat, observed in this study. Further studies on the CMO effects on maternal metabolism and pup development, are required to define the mechanisms by which the physiological responses of the dam impair offspring

development, metabolism and GIT microbial composition. A secondary study where pups receive the same maternal diet for 30 days after weaning would be also important to define the effects of mismatch between maternal environment and adult life.

Even though CMO contain oligosaccharides similar to HMO, the present study was not able to demonstrate its prebiotic effects. A prebiotic is defined as a food ingredient able to: (1) resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (2) be fermented by the intestinal microbiota; (3) stimulate selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing. Although CMO were shown to be able to be fermented by selected bifidobacteria a CMO enriched diet was unable to stimulate growth and/or activity of commensal bacteria in the GIT of the dams. Positives effects of dams CMO intake on the microbiota composition of the pups at weaning; however, was shown. It may suggest that the definition of prebiotic should consider the effects of maternal dietary intake on the pup microbiota. In addition, molecular-based microbiological methodologies, such as metatrancriptomics, and other methods such as metabolomics have been developed and should be applied to the identification of prebiotics. Regarding the stimulation of bacterial activity, for example, the patterns of production of organic acids, gases and enzymes have been used. However, these have not been validated as yet as biomarkers of specific bacterial genera.

6.4 Concluding remarks

The present study is the first to report New Zealand Saanen caprine colostrum, milk and whey composition which contain oligosaccharides similar to HMO. A method of enrichment previously described was used to produce a CMOP for *in vitro* and *in vivo* characterisation of its health effects. CMOP was shown to contain high concentrations of sialyloligosaccharides, known for their health benefits, and for the first time, shown to differentially stimulate the growth of bifidobacteria, commonly found in the GIT of breast-fed infants. Among the bifidobacterial species tested, *B. bifidum* utilised CMO most efficiently when compared to *B.*

breve and *B. longum* subsp*. longum. B. bifidum* (AGR2166) was shown to ferment the sialyloligosaccharides, 3'- and 6'-sialyl-lactose present in CMOP through cell-associated sialidase expression. Augmented microbial biomass associated with enhanced growth and *in vitro* fermentation of CMOP, increased the production of microbial fermentation end products such as acetate and lactate. These findings indicate that CMO consumption by infants may stimulate the growth and fermentation of GIT bifidobacteria population.

GF and conventional rodent models were used to test the effects of maternal CMO consumption during pregnancy and the effects on the offspring. In the first study, GF mice or GF mice mono-associated with *B. bifidum* (AGR2166) were fed CMO during pregnancy. CMO diet showed no effects on maternal GIT or foetal growth. Mice inoculated with *B. bifidum* (AGR2166) and fed CMO diet, however, showed an increased bacterial translocation from maternal GIT to organs and placenta as indicated by the presence of bacterial DNA. Increased translocation of commensal bacteria from maternal GIT to the foetus may have effects on foetal immunological programming.

Testing the effects of CMO on complex GIT microbiota of conventional mice, also failed to demonstrate any important effects on the maternal GIT. However, effects on maternal lipid metabolism and milk protein concentration were shown to improve pups development and increase numbers of bifidobacteria in the colon at weaning. The CMO effects on maternal lipid metabolism were observed in pups at weaning and also in pups consuming control diet 30 days after weaning. Increased body fat, however was observed in pups 30 days after weaning. In conclusion, dietary CMO impact the bifidobacterial component of the neonatal GIT microbiota through indirect effects of maternal supplementation during pregnancy. Supplementation of maternal diet may also positively impact the growth of the pups up to weaning, with further studies still required to assess whether maternally derived effects are maintained long-term.

Appendices

Appendix A. Percentage 16S rRNA sequences in dam colon digesta.

Depth	Identification	Control	Combo	CMO	P-value	FDR
Phylum	Tenericutes	0.13	0.93	0.65	0.18	1.00
	Proteobacteria	2.23	5.43	2.42	0.43	1.00
	Unclassified Bacteria	0.76	0.71	0.49	0.49	1.00
	Actinobacteria	0.11	0.24	0.07	0.83	1.00
	Bacteroidetes	63.66	59.09	60.84	0.97	1.00
	Firmicutes	33.11	33.55	35.53	1.00	1.00
Family	Porphyromonadaceae	41.39[a]	11.97[b]	28.28[b]	0.06	0.55
	Ruminococcaceae	3.96	1.06	5.59	0.12	0.55
	Unclassified Proteobacteria	0.29	0.63	1.30	0.12	0.55
	Unclassified Clostridia	0.02	0.01	0.06	0.16	0.55
	Unclassified Firmicutes	0.24	0.41	0.17	0.18	0.55
	Mycoplasmataceae	0.10	0.93	0.61	0.18	0.55
	Bacteroidaceae	10.74	35.17	2.78	0.21	0.55
	Erysipelotrichaceae	0.52	4.25	2.37	0.21	0.55
	Rikenellaceae	5.30	2.31	13.58	0.21	0.55
	Unclassified Bacilli	0.02	0.01	0.00	0.22	0.55
	Helicobacteraceae	0.38	0.04	0.00	0.23	0.55

	Lactobacillaceae	3.80	7.51	0.33	0.25	0.55
	Unclassified Lactobacillales	0.66	1.07	0.06	0.25	0.55
	Clostridiaceae	0.15	0.41	0.21	0.32	0.60
	Unclassified Desulfovibrionales	0.26	0.32	0.74	0.35	0.60
	Lachnospiraceae	9.04	2.81	9.59	0.36	0.60
	Unclassified Bacteroidetes	4.38	7.65	14.57	0.36	0.60
	Unclassified Bacteria	0.76	0.71	0.49	0.49	0.74
	Streptococcaceae	0.22	0.24	0.05	0.50	0.74
	Enterobacteriaceae	1.24	4.38	0.01	0.55	0.77
	Unclassified Clostridiales	13.11	7.85	15.12	0.61	0.77
	Peptostreptococcaceae	1.26	7.87	1.86	0.62	0.77
	Staphylococcaceae	0.08	0.04	0.04	0.63	0.77
	Incertae Sedis XIII	0.01	0.01	0.02	0.75	0.88
	Bifidobacteriaceae	0.01	0.12	0.00	0.78	0.88
	Desulfovibrionaceae	0.02	0.01	0.29	0.81	0.88
	Coriobacteriaceae	0.10	0.12	0.07	0.88	0.92
	Unclassified Bacteroidales	1.85	1.98	1.64	0.97	0.97
	Odoribacter	0.45 [a]	0.62 [a]	1.83 [b]	0.02	0.54
	Lactococcus	0.02 [a]	0.14 [b]	0.05 [a]	0.05	0.54
Genus	Unclassified Porphyromonadaceae	38.17 [a]	10.70 [b]	22.14 [b]	0.05	0.54
	Oscillibacter	0.70 [a]	0.11 [a]	1.52 [b]	0.07	0.54
	Unclassified Clostridia	0.02 [a]	0.01 [a]	0.06 [b]	0.07	0.54

Allobaculum	0.27 [a]	3.89 [b]	1.97 [ab]	0.08	0.54
Ureaplasma	0.08	0.92	0.61	0.11	0.54
Bifidobacterium	0.01	0.12	0.00	0.12	0.54
Unclassified Erysipelotrichaceae	0.03	0.19	0.20	0.13	0.54
Robinsoniella	0.23	1.04	2.31	0.14	0.54
Unclassified Coriobacteriaceae	0.00	0.01	0.02	0.15	0.54
Sporacetigenium	0.58	3.30	0.69	0.15	0.54
Unclassified Peptostreptococcaceae	0.68	4.57	1.17	0.16	0.54
Ethanoligenens	0.01	0.00	0.05	0.16	0.54
Unclassified Proteobacteria	0.29	0.63	1.30	0.16	0.54
Unclassified Firmicutes	0.24	0.41	0.17	0.17	0.54
Bacteroides	10.74	35.17	2.78	0.18	0.54
Alistipes	5.07	2.18	12.89	0.22	0.63
Rikenella	0.17	0.09	0.65	0.29	0.69
Unclassified Ruminococcaceae	3.18	0.90	3.86	0.31	0.69
Unclassified Bacteroidetes	4.38	7.65	14.57	0.33	0.69
Lactobacillus	2.89	5.77	0.23	0.36	0.69
Unclassified Desulfovibrionales	0.26	0.32	0.74	0.37	0.69
Clostridium	0.08	0.23	0.14	0.37	0.69
Unclassified Enterobacteriaceae	0.74	2.90	0.00	0.38	0.69
Unclassified Lactobacillales	0.66	1.07	0.06	0.39	0.69
Unclassified Lactobacillaceae	0.90	1.74	0.10	0.39	0.69
Sporobacter	0.01	0.00	0.02	0.40	0.69

Unclassified Clostridiaceae	0.07	0.18	0.07	0.42	0.69
Papillibacter	0.04	0.05	0.10	0.45	0.69
Helicobacter	0.36	0.04	0.00	0.46	0.69
Parabacteroides	0.76	0.04	1.87	0.46	0.69
Dorea	1.88	0.15	1.18	0.49	0.69
Escherichia/Shigella	0.50	1.48	0.00	0.49	0.69
Unclassified Lachnospiraceae	5.90	1.60	5.73	0.50	0.69
Unclassified Bacilli	0.02	0.01	0.00	0.50	0.69
Roseburia	0.73	0.01	0.18	0.51	0.69
Streptococcus	0.20	0.10	0.00	0.54	0.71
Anaerovorax	0.01	0.00	0.02	0.55	0.71
Barnesiella	2.00	0.61	2.44	0.57	0.71
Unclassified Bacteria	0.76	0.71	0.49	0.58	0.71
Anaerotruncus	0.01	0.00	0.03	0.60	0.71
Gemella	0.08	0.04	0.04	0.61	0.71
Enterorhabdus	0.09	0.09	0.03	0.65	0.74
Johnsonella	0.31	0.01	0.18	0.67	0.75
Unclassified Clostridiales	13.11	7.85	15.12	0.75	0.82
Unclassified Rikenellaceae	0.06	0.03	0.03	0.77	0.83
Unclassified Desulfovibrionaceae	0.02	0.01	0.02	0.87	0.91
Turicibacter	0.22	0.12	0.20	0.91	0.94
Unclassified Bacteroidales	1.85	1.98	1.64	0.96	0.97

Dams (n = Control, 6; Combo, 2; CMO, 2).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

FDR, false discovery rate.

Appendix B. Percentage 16S rRNA sequences in pup colon digesta at weaning.

Depth	Identification	Control	Combo	CMO	P-value	FDR
Phylum	Unclassified Bacteria	1.39	1.22	1.82	0.19	0.96
	Verrucomicrobia	0.00	0.00	0.00	0.59	0.96
	Proteobacteria	2.76	2.76	3.84	0.65	0.96
	Deferribacteres	0.02	0.11	0.09	0.67	0.96
	Tenericutes	0.34	0.29	0.18	0.76	0.96
	Bacteroidetes	56.75	53.51	54.84	0.88	0.96
	Firmicutes	38.62	41.97	37.64	0.92	0.96
	Actinobacteria	0.11	0.14	1.58	0.96	0.96
Family	Unclassified Epsilonproteobacteria	0.000	0.001	0.002	0.07	0.96
	Carnobacteriaceae	0.000	0.003	0.000	0.14	0.96
	Unclassified Desulfovibrionales	0.62	0.37	0.98	0.14	0.96
	Desulfovibrionaceae	0.05	0.02	0.06	0.14	0.96
	Unclassified Bacillales	0.000	0.006	0.000	0.14	0.96
	Staphylococcaceae	0.04	0.17	0.02	0.16	0.96
	Unclassified Bacteria	1.39	1.22	1.82	0.20	0.96
	Unclassified Proteobacteria	0.95	0.59	1.66	0.22	0.96
	Ruminococcaceae	3.16	4.70	4.12	0.23	0.96
	Streptococcaceae	0.24	0.32	0.17	0.24	0.96
	Unclassified Gammaproteobacteria	0.002	0.001	0.004	0.25	0.96
	Anaeroplasmataceae	0.01	0.04	0.01	0.28	0.97

Unclassified Deltaproteobacteria	0.01	0.01	0.01	0.33	1.00
Helicobacteraceae	0.80	1.40	0.89	0.41	1.00
Peptostreptococcaceae	5.35	2.29	1.03	0.46	1.00
Rikenellaceae	7.68	8.39	13.70	0.46	1.00
Unclassified Bacteroidales	2.15	1.79	1.79	0.47	1.00
Unclassified Bacteroidetes	4.65	9.05	7.36	0.52	1.00
Porphyromonadaceae	30.69	25.81	28.83	0.53	1.00
Bifidobacteriaceae	0.06	0.08	1.50	0.56	1.00
Incertae Sedis XIII	0.01	0.01	0.02	0.56	1.00
Eubacteriaceae	0.001	0.003	0.001	0.59	1.00
Unclassified Clostridia	0.05	0.07	0.06	0.59	1.00
Verrucomicrobiaceae	0.001	0.002	0.003	0.59	1.00
Erysipelotrichaceae	5.95	7.59	8.90	0.60	1.00
Deferribacteraceae	0.02	0.11	0.09	0.67	1.00
Unclassified Clostridiales	12.51	14.20	12.04	0.77	1.00
Bacteroidaceae	11.57	8.47	3.15	0.77	1.00
Unclassified Lactobacillales	0.28	0.38	0.19	0.77	1.00
Lactobacillaceae	1.51	2.23	1.02	0.78	1.00
Enterococcaceae	0.002	0.003	0.004	0.78	1.00
Lachnospiraceae	8.50	8.58	8.95	0.79	1.00
Incertae Sedis XIV	0.003	0.004	0.003	0.83	1.00
Peptococcaceae	0.01	0.01	0.01	0.89	1.00

	Unclassified Bacilli	0.01	0.01	0.01	0.89	1.00
	Coriobacteriaceae	0.05	0.05	0.08	0.92	1.00
	Alcaligenaceae	0.08	0.03	0.14	0.94	1.00
	Enterobacteriaceae	0.24	0.14	0.09	0.95	1.00
	Unclassified Firmicutes	0.54	0.58	0.62	0.95	1.00
	Mycoplasmataceae	0.34	0.25	0.17	0.96	1.00
	Clostridiaceae	0.46	0.81	0.49	0.99	1.00
	Pasteurellaceae	0.01	0.18	0.00	1.00	1.00
	Bifidobacterium	0.05 [a]	0.07 [a]	1.37 [b]	0.03	0.86
	Unclassified Bifidobacteriaceae	0.01 [a]	0.01 [a]	0.13 [b]	0.04	0.86
	Parabacteroides	1.51 [a]	0.48 [a]	4.51 [b]	0.04	0.86
	Barnesiella	3.34 [a]	1.36 [b]	2.18 [ab]	0.05	0.86
	Unclassified Epsilonproteobacteria	0.0000	0.0007	0.002	0.07	0.86
	Unclassified Ruminococcaceae	2.38	3.93	3.33	0.10	0.86
	Alistipes	7.37	7.24	13.05	0.11	0.86
	Unclassified Erysipelotrichaceae	1.36	1.20	3.98	0.12	0.86
	Coprobacillus	0.06	0.72	0.44	0.12	0.86
	Anaerosporobacter	0.001	0.0003	0.003	0.12	0.86
	Unclassified Bacteria	1.39	1.22	1.82	0.13	0.86
	Granulicatella	0.0004	0.003	0.00	0.14	0.86
	Unclassified Streptococcaceae	0.0004	0.002	0.003	0.15	0.86
Genus	Unclassified Helicobacteraceae	0.04	0.08	0.04	0.17	0.86

Anaeroplasma	0.01	0.04	0.01	0.18	0.86
Roseburia	0.21	0.71	0.30	0.18	0.86
Streptococcus	0.03	0.13	0.02	0.19	0.86
Olsenella	0.004	0.001	0.04	0.20	0.86
Anaerotruncus	0.02	0.04	0.03	0.20	0.86
Helicobacter	0.77	1.33	0.85	0.26	0.86
Tannerella	0.00	0.00	0.01	0.27	0.86
Hydrogenoanaerobacterium	0.00	0.00	0.00	0.27	0.86
Unclassified Bacillales	0.00	0.01	0.00	0.28	0.86
Bacteroides	11.57	8.47	3.15	0.29	0.86
Desulfovibrio	0.03	0.00	0.02	0.30	0.86
Unclassified Desulfovibrionales	0.62	0.37	0.98	0.31	0.86
Unclassified Mycoplasmataceae	0.00	0.00	0.00	0.33	0.86
Unclassified Clostridia	0.05	0.07	0.06	0.33	0.86
Unclassified Gammaproteobacteria	0.00	0.00	0.00	0.34	0.86
Acetivibrio	0.00	0.01	0.01	0.36	0.86
Unclassified Proteobacteria	0.95	0.59	1.66	0.36	0.86
Gemella	0.04	0.17	0.02	0.38	0.86
Unclassified Bacteroidetes	4.65	9.05	7.36	0.39	0.86
Unclassified Deltaproteobacteria	0.01	0.01	0.01	0.42	0.86
Unclassified Pasteurellaceae	0.00	0.13	0.00	0.42	0.86
Unclassified Coriobacteriaceae	0.01	0.01	0.00	0.42	0.86

Pasteurella	0.00	0.06	0.00	0.44	0.86
Papillibacter	0.04	0.05	0.05	0.44	0.86
Unclassified Lactobacillaceae	0.29	0.50	0.22	0.45	0.86
Blautia	0.00	0.00	0.00	0.46	0.86
Unclassified Enterobacteriaceae	0.17	0.10	0.06	0.47	0.86
Eubacterium	0.00	0.00	0.00	0.48	0.86
Unclassified Peptostreptococcaceae	3.15	1.24	0.57	0.49	0.86
Unclassified Lactobacillales	0.28	0.38	0.19	0.49	0.86
Anaerovorax	0.01	0.01	0.02	0.51	0.86
Sporacetigenium	2.20	1.05	0.46	0.52	0.86
Rikenella	0.21	1.06	0.52	0.53	0.86
Escherichia/Shigella	0.07	0.05	0.03	0.53	0.86
Mucispirillum	0.02	0.11	0.09	0.54	0.86
Lactobacillus	1.22	1.73	0.80	0.55	0.86
Clostridium	0.23	0.42	0.24	0.55	0.86
Turicibacter	0.34	0.49	0.16	0.57	0.87
Unclassified Bacteroidales	2.15	1.79	1.79	0.58	0.87
Akkermansia	0.00	0.00	0.00	0.59	0.87
Parasutterella	0.08	0.03	0.14	0.61	0.87
Odoribacter	1.16	0.89	1.09	0.62	0.87
Ethanoligenens	0.01	0.01	0.00	0.63	0.87
Unclassified Clostridiaceae	0.22	0.38	0.24	0.63	0.87

Unclassified Rikenellaceae	0.11	0.10	0.13	0.64	0.87
Ruminococcus	0.00	0.01	0.00	0.66	0.87
Enterorhabdus	0.04	0.05	0.03	0.68	0.89
Sarcina	0.00	0.01	0.00	0.70	0.90
Dorea	0.88	0.69	0.96	0.72	0.91
Unclassified Desulfovibrionaceae	0.02	0.02	0.03	0.73	0.91
Robinsoniella	1.11	0.74	0.71	0.75	0.91
Johnsonella	0.19	0.12	0.13	0.76	0.91
Ureaplasma	0.34	0.23	0.17	0.77	0.91
Unclassified Clostridiales	12.51	14.20	12.04	0.79	0.93
Lactococcus	0.21	0.19	0.15	0.84	0.94
Enterococcus	0.00	0.00	0.00	0.86	0.94
Holdemania	0.03	0.03	0.04	0.88	0.94
Unclassified Peptococcaceae	0.00	0.00	0.00	0.89	0.94
Unclassified Firmicutes	0.54	0.58	0.62	0.89	0.94
Allobaculum	4.16	5.15	4.27	0.89	0.94
Sporobacter	0.01	0.01	0.01	0.90	0.94
Unclassified Lachnospiraceae	6.12	6.32	6.85	0.91	0.94
Unclassified Porphyromonadaceae	24.68	23.07	21.06	0.91	0.94
Oscillibacter	0.69	0.64	0.68	0.95	0.97
Peptococcus	0.00	0.00	0.01	0.97	0.99
Unclassified Bacilli	0.01	0.01	0.01	0.99	0.99

Pup weaning (n = Control, 19; Combo, 20; CMO, 13).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

FDR, false discovery rate.

Appendix C. Percentage 16S rRNA sequences in pup colon digesta 30 days after weaning.

Depth	Description	Control	Combo	CMO	P-value	FDR
	Unclassified	0.000 [a]	0.001 [a]	0.004 [b]	0.06	0.61
	Unclassified Bacteria	1.51	1.13	1.64	0.20	0.72
	Deferribacteres	0.24	0.13	0.09	0.24	0.72
	Tenericutes	0.16	0.32	0.16	0.33	0.74
Phylum	Firmicutes	36.68	40.70	45.80	0.44	0.75
	Actinobacteria	0.69	0.10	0.31	0.50	0.75
	Bacteroidetes	56.27	52.61	48.95	0.67	0.78
	Proteobacteria	4.40	3.38	3.02	0.70	0.78
	Verrucomicrobia	0.04	1.63	0.02	0.78	0.78
	Incertae Sedis XIV	0.001 [a]	0.012 [b]	0.001 [a]	0.0001	0.01
	Peptostreptococcaceae	0.58 [a]	4.67 [b]	1.13 [a]	0.005	0.12
	Clostridiaceae	0.32 [a]	0.73 [b]	0.19 [a]	0.03	0.44
	Rikenellaceae	9.05 [a]	7.49 [ab]	5.60 [b]	0.04	0.49
	Unclassified	0.000 [a]	0.001 [a]	0.004 [b]	0.06	0.57
Family	Streptococcaceae	0.16	0.24	0.10	0.12	0.76
	Peptococcaceae	0.02	0.00	0.01	0.18	0.76
	Unclassified Bacteria	1.51	1.13	1.64	0.20	0.76
	Enterobacteriaceae	0.08	0.31	0.08	0.22	0.76
	Alcaligenaceae	0.15	0.00	0.01	0.23	0.76
	Unclassified Firmicutes	0.58	0.38	0.94	0.23	0.76

Deferribacteraceae	0.24	0.13	0.09	0.24	0.76
Mycoplasmataceae	0.15	0.32	0.15	0.24	0.76
Porphyromonadaceae	34.19	25.06	28.08	0.25	0.76
Unclassified Desulfovibrionales	0.99	0.59	0.64	0.33	0.85
Lactobacillaceae	1.58	0.67	0.81	0.34	0.85
Unclassified Bacilli	0.01	0.00	0.01	0.38	0.85
Unclassified Bacteroidales	1.94	1.62	1.46	0.40	0.85
Helicobacteraceae	1.80	1.31	0.95	0.40	0.85
Unclassified Alphaproteobacteria	0.00	0.01	0.00	0.44	0.85
Unclassified Lactobacillales	0.29	0.12	0.16	0.45	0.85
Pasteurellaceae	0.00	0.01	0.01	0.46	0.85
Bifidobacteriaceae	0.64	0.04	0.27	0.46	0.85
Unclassified Clostridiales	10.61	12.51	13.48	0.56	0.97
Erysipelotrichaceae	8.57	6.87	14.32	0.59	0.98
Unclassified Bacteroidetes	5.66	11.07	7.35	0.62	0.98
Anaeroplasmataceae	0.01	0.01	0.01	0.64	0.98
Enterococcaceae	0.01	0.00	0.00	0.67	0.98
Unclassified Gammaproteobacteria	0.00	0.00	0.00	0.73	0.98
Unclassified Burkholderiales	0.01	0.00	0.00	0.73	0.98
Verrucomicrobiaceae	0.04	1.63	0.02	0.78	0.98
Desulfovibrionaceae	0.13	0.17	0.25	0.79	0.98
Unclassified Proteobacteria	1.22	0.96	1.05	0.83	0.98

	Incertae Sedis XIII	0.01	0.01	0.01	0.86	0.98
	Bacteroidaceae	5.44	7.37	6.46	0.87	0.98
	Unclassified Deltaproteobacteria	0.01	0.01	0.01	0.87	0.98
	Unclassified Clostridia	0.05	0.05	0.04	0.88	0.98
	Eubacteriaceae	0.00	0.00	0.00	0.89	0.98
	Lachnospiraceae	9.22	9.98	10.43	0.93	0.99
	Staphylococcaceae	0.04	0.03	0.02	0.97	0.99
	Coriobacteriaceae	0.05	0.06	0.05	0.97	0.99
	Ruminococcaceae	4.63	4.41	4.16	1.00	1.00
	Blautia	0.0004 [a]	0.01 [b]	0.00 [a]	0.001	0.08
	Turicibacter	0.09 [a]	1.69 [b]	0.12 [a]	0.007	0.26
	Unclassified Peptostreptococcaceae	0.32 [a]	2.71 [b]	0.64 [a]	0.01	0.26
	Sporacetigenium	0.26 [a]	1.96 [b]	0.49 [ab]	0.01	0.26
	Unclassified Firmicutes	0.58 [ab]	0.38 [a]	0.94 [b]	0.01	0.26
	Unclassified Clostridiaceae	0.16 [a]	0.36 [b]	0.08 [a]	0.02	0.29
Genus	Alistipes	8.23 [a]	7.11 [ab]	5.15 [b]	0.04	0.78
	Clostridium	0.17 [a]	0.37 [b]	0.10 [a]	0.04	0.49
	Allobaculum	5.56 [a]	3.09 [a]	10.78 [b]	0.05	0.54
	Barnesiella	3.99 [a]	1.80 [b]	1.88 [b]	0.07	0.58
	Parabacteroides	6.32 [a]	1.55 [b]	1.71 [b]	0.07	0.58
	Unclassified	0.000 [a]	0.001 [a]	0.004 [b]	0.07	0.58
	Unclassified Bacteria	1.51	1.13	1.64	0.09	0.63

	Unclassified Desulfovibrionaceae	0.08	0.02	0.02	0.09	0.63
	Unclassified Lactobacillaceae	0.36	0.16	0.15	0.11	0.66
	Lactobacillus	1.22	0.50	0.66	0.12	0.66
	Lactococcus	0.10	0.21	0.08	0.13	0.66
	Peptococcus	0.01	0.00	0.01	0.14	0.66
	Unclassified Bacteroidetes	5.66	11.07	7.35	0.15	0.66
	Unclassified Lactobacillales	0.29	0.12	0.16	0.16	0.66
	Unclassified Rikenellaceae	0.14	0.09	0.07	0.16	0.66
	Akkermansia	0.04	1.63	0.02	0.18	0.70
	Rikenella	0.68	0.29	0.38	0.19	0.70
	Parasutterella	0.15	0.00	0.01	0.23	0.78
	Unclassified Peptococcaceae	0.00	0.00	0.00	0.23	0.78
	Marvinbryantia	0.00	0.00	0.00	0.24	0.78
	Unclassified Erysipelotrichaceae	2.76	1.21	3.31	0.27	0.78
	Ureaplasma	0.15	0.32	0.15	0.28	0.78
	Holdemania	0.01	0.00	0.01	0.29	0.78
	Unclassified Alphaproteobacteria	0.00	0.01	0.00	0.31	0.78
	Helicobacter	1.72	1.26	0.90	0.32	0.78
	Coprobacillus	0.15	0.88	0.10	0.32	0.78
	Desulfovibrio	0.05	0.15	0.23	0.33	0.78
	Ethanoligenens	0.00	0.00	0.02	0.34	0.78
	Bifidobacterium	0.58	0.04	0.24	0.34	0.78

	Oscillibacter	0.74	0.55	0.81	0.34	0.78
	Unclassified Bifidobacteriaceae	0.05	0.00	0.02	0.35	0.78
	Unclassified Burkholderiales	0.01	0.00	0.00	0.37	0.78
	Unclassified Bacteroidales	1.94	1.62	1.46	0.38	0.78
	Unclassified Desulfovibrionales	0.99	0.59	0.64	0.38	0.78
	Unclassified Bacilli	0.01	0.00	0.01	0.40	0.78
	Unclassified Helicobacteraceae	0.08	0.05	0.05	0.40	0.78
	Odoribacter	1.45	1.29	1.05	0.40	0.78
	Streptococcus	0.06	0.03	0.02	0.42	0.78
	Robinsoniella	0.84	0.54	1.48	0.44	0.78
	Hydrogenoanaerobacterium	0.00	0.00	0.01	0.44	0.78
	Unclassified Alcaligenaceae	0.00	0.00	0.00	0.45	0.78
	Enterorhabdus	0.03	0.04	0.02	0.46	0.79
	Gemella	0.04	0.03	0.02	0.51	0.82
	Ruminococcus	0.00	0.00	0.00	0.52	0.82
	Anaerosporobacter	0.00	0.00	0.00	0.53	0.82
	Mucispirillum	0.24	0.13	0.09	0.58	0.82
	Unclassified Gammaproteobacteria	0.00	0.00	0.00	0.59	0.82
	Unclassified Enterobacteriaceae	0.05	0.20	0.05	0.59	0.82
	Anaerotruncus	0.03	0.03	0.02	0.59	0.82
	Escherichia/Shigella	0.03	0.11	0.03	0.59	0.82
	Unclassified Pasteurellaceae	0.00	0.01	0.01	0.59	0.82

	Anaerovorax	0.01	0.01	0.01	0.60	0.82
	Roseburia	0.14	0.30	0.38	0.62	0.82
	Unclassified Clostridiales	10.61	12.51	13.48	0.62	0.82
	Unclassified Clostridiales Incertae Sedis XIII	0.00	0.00	0.00	0.62	0.82
	Sporobacter	0.01	0.01	0.01	0.63	0.82
	Olsenella	0.01	0.01	0.02	0.64	0.82
	Johnsonella	0.32	0.46	0.13	0.64	0.82
	Unclassified Clostridia	0.05	0.05	0.04	0.65	0.82
	Acetivibrio	0.01	0.01	0.01	0.70	0.86
	Anaeroplasma	0.01	0.01	0.01	0.70	0.86
	Dorea	0.95	1.09	0.81	0.73	0.86
	Coprococcus	0.00	0.00	0.00	0.73	0.86
	Enterococcus	0.00	0.00	0.00	0.74	0.86
	Unclassified Coriobacteriaceae	0.01	0.01	0.01	0.76	0.86
	Unclassified Ruminococcaceae	3.77	3.74	3.22	0.76	0.86
	Pasteurella	0.00	0.01	0.00	0.77	0.86
	Unclassified Porphyromonadaceae	22.42	20.42	23.44	0.88	0.96
	Bacteroides	5.44	7.37	6.46	0.89	0.96
	Unclassified Proteobacteria	1.22	0.96	1.05	0.89	0.96
	Unclassified Lachnospiraceae	6.95	7.57	7.63	0.92	0.97
	Papillibacter	0.05	0.06	0.06	0.93	0.97
	Tannerella	0.00	0.00	0.00	0.94	0.97

	Eubacterium	0.00	0.00	0.00	0.97	0.98
	Sarcina	0.00	0.00	0.00	0.97	0.98
	Unclassified Deltaproteobacteria	0.01	0.01	0.01	0.99	0.99

Pup 30 days after weaning (n = Control, 18; Combo, 18; CMO, 16).

[a, b, c] Values with similar letters in rows do not differ significantly ($P < 0.05$).

FDR, false discovery rate.

Appendix D. Annotated urine metabolites from LC-MS analysis in HILIC and C18 columns negative and positive mode found to separate dams according to the treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
HILIC/ +	81.0438	0.07		↑ Combo X Control; CMO	Acetone	M+Na	81.0311
HILIC/ +	101.0711	0.05		↑ Combo X Control; CMO	Hexanal	M+H	101.0961
					Senecioic acid	M+H	101.0597
HILIC/ +	148.1328	0.03		↓ Combo X Control; CMO	D-Glutamic acid	M+H	148.0604
					Glutaconic acid	M+NH4	148.0604
HILIC/ +	180.0512	0.02		↓ CMO X Control; Combo	Hippuric acid	M+H	180.0655
HILIC/ +	214.0906	0.009		↑ Combo X Control; CMO	Homoveratric acid	M+NH4	214.1074
HILIC/ +	274.0921	0.004	0.9	↓ CMO; Combo X Control	L-Thyronine	M+H	274.1074
					Glutaconylcarnitine	M+H	274.1285
HILIC/ +	293.0629	0.003	0.9	↑ Combo X Control; CMO	Phenylglucuronide	M+Na	293.0632
					Carboxyphosphamide	M+H	293.0219
					Estrone	M+Na	293.1512
					Canavaninosuccinate	M+H	293.1092
HILIC/ +	303.1772	0.03		↓ Combo X Control; CMO	Linoleic acid	M+Na	303.2294
HILIC/ +	544.7291	0.03		↑ CMO X Control; Combo	glycerophospholipid	M+H	
HILIC/ +	736.5043	0.02		↑ Combo X Control; CMO	glycerophospholipid	M+H	
C18/-	164.0400	0.004	0.9	↓ CMO; Combo X Control	Methionine sulfoxide	M-H	164.0386
C18/+	83.0597	0.007	0.1	↑ CMO X Combo	Urea	M+Na	83.0215

C18/+	119.0838	0.006	0.1	↑ Combo X Control; CMO	2-Hydroxy-3-methylbutyric acid	M+H	119.0921
C18/+	153.1250	0.005	0.1	↑ Combo X Control; CMO	L-Octanoylcarnitine	M+H+NH4	153.1253
C18/+	155.1405	0.001	0.1	↑ Combo X Control; CMO	m-Tyramine	M+NH4	155.1178
					1-Methylnicotinamide	M+NH4	155.1053
					Ornithine	M+Na	155.0790
C18/+	179.1394	0.001	0.1	↑ CMO X Control; Combo	L-Carnitine	M+NH4	179.1390
C18/+	184.1295	0.003	0.1	↑ Combo X Control; CMO	5-Aminoimidazole	2M+NH4	184.1305
					5a-Tetrahydrocortisol	M+2H	184.1275
C18/+	200.2003	0.006	0.1	↑ Combo X Control; CMO	Sorbitol	M+NH4	200.1128
C18/+	213.1479	0.002	0.1	↑ Combo X Control; CMO	3a,6b,7a,12a-Tetrahydroxy-5b-cholanoic acid	M+2H	213.1485
					Other bile acids	M+2H	214.1485
C18/+	255.1570	0.003	0.1	↑ Combo X Control; CMO	Histidinyl-Valine	M+1H	254.2856
C18/+	308.2937	0.0006	0.1	↑ Combo X Control; CMO	Epiandrosterone	M+NH4	308.2584
					Etiocholanolone	M+NH4	308.2584
C18/+	311.2543	0.007	0.1	↑ CMO X Combo	16b-Hydroxyestradiol	M+Na	311.1617
					17-Epiestriol	M+Na	311.1617
C18/+	331.2077	0.007	0.1	↑ Combo X Control; CMO	17-Hydroxyprogesterone	M+H	331.2267
C18/+	337.3285	0.006	0.1	↓ Combo X Control; CMO	Palmitoleic acid	M+2ACN+H	337.2849

C18/+	346.2695	0.001	0.1	↑ Combo X Control; CMO	Diacylglycerols	M+2H	346.2716
					Docosahexaenoic acid	M+NH4	346.2740
C18/+	348.2769	0.002	0.1	↑ Combo X Control; CMO	17-Hydroxyprogesterone	M+NH4	348.2533
					6(beta)-hydroxyprogesterone	M+NH4	348.2533
C18/+	394.3270	0.004	0.1	↑ Combo X Control; CMO	12b-Hydroxy-5b-cholanoic acid	M+NH4	394.3315
					Allolithocholic acid	M+NH4	394.3315
					Lithocholic acid	M+NH4	394.3315
C18/+	477.3647	0.0003	0.1	↑ Combo X Control; CMO	27-Norcholestanehexol	M+Na	477.3186

Appendix E Annotated urine metabolites from liquid chromatography-mass spectrometry analysis in HILIC and C18 columns negative and positive mode found to separate pup 30 days after weaning accordingly with dams treatment.

Column/Mode	MLCMS [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
HILIC/-	160.0606	0.006	0.9	↑ Combo X Control; CMO	Aminoadipic acid	M-H	161.0688
HILIC/-	196.0722	0.003	0.9	↓ CMO X Control; Combo	Citrulline	M+Na-2H	196.0704
HILIC/-	219.9999	0.002	0.9	↑ Combo X Control; CMO	O-Phosphohomoserine	M+Na-2H	219.9992
					O-Phosphothreonine	M+Na-2H	219.9992
HILIC/+	154.0608	0.0001		↑ Combo X Control; CMO	Creatinine	M+Na	154.0587
HILIC/+	224.0581	0.003	0.6	↓ CMO; Combo X Control	Homocitric acid	M+NH4	224.0765
HILIC/+	263.0872	0.001	0.6	↓ CMO; Combo X Control	L-beta-aspartyl-L-glutamic acid	M+H	263.0874
HILIC/+	267.0734	0.06		↑ Combo X CMO	Indolylacryloylglycine	M+Na	267.0740
HILIC/+	291.0443	0.02		↑ Combo X CMO	Argininosuccinic acid	M+H	291.1299
HILIC/+	308.2216	0.006		↑ Combo X CMO	Epiandrosterone	M+NH4	308.2584
					Etiocholanolone	M+NH4	308.2584
HILIC/+	319.0654	0.001	0.6	↓ CMO; Combo X Control	4-Hydroxyenterodiol	M+H	319.1540
HILIC/+	337.2122	0.02		↑ Combo X CMO	7-Dehydropregnenolone	M+Na	337.2138
HILIC/+	694.4949	0.01		↑ Combo X CMO	Glycerophospholipid		
C18/-	85.0638	0.004	0.8	↓ CMO; Combo X Control	Iso-Valeraldehyde	M-H	86.0732
					But-2-enoic acid	M-H	85.0295

C18/-	92.0562	0.003	0.8	↓ CMO X Control; Combo	Phenol	M-H	94.0419
					3-Oxodecanoic acid	M-2H	58.0419
C18/-	107.0477	0.0003	0.5	↓ CMO; Combo X Control	p-Cresol	M-H	108.0575
					(R)-3-Hydroxydodecanoic acid	M-2H	107.079
C18/-	115.9183	0.060		↑ Combo X Control; CMO	Homocysteine thiolactone	M-H	116.0176
C18/-	164.04	0.004	0.9	↓ CMO; Combo X Control	Methionine sulfoxide	M-H	164.0387
C18/-	172.0932	0.004	0.8	Control - CMO	N-Acetyl-L-glutamate 5-semialdehyde	M-H	172.0615
					Quinaldic acid	M-H	172.0404
C18/-	193.0495	0.04		↑ Combo X CMO	Vanillactic acid	M-H20-H	212.0685
C18/-	242.1357	0.004	0.8	↑ Combo X Control; CMO	Tiglylcarnitine	M-H	242.1398
C18/+	301.1398	0.05		↑ CMO X Control; Combo	5-Nonyltetrahydro-2-oxo-3-furancarboxylic acid	M+2Na-H	301.1386

Appendix F. Annotated plasma metabolites from liquid chromatography-mass spectrometry analysis in HILIC and C18 columns, negative and positive mode, found to separate dams according to the treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
HILIC/ +	160.0970[#]	8E-05	0.08	↑ CMO X Control; Combo	1-Methylnicotinamide	M+Na	160.0607
					3-Dehydrocarnitine	M+H	160.0968
HILIC/ +	341.1582	2E-03	0.7	↑ CMO X Control; Combo	3'-Ketolactose	M+H	341.1078
HILIC/ +	205.1550	2E-03	0.7	↑ CMO X Control; Combo	N-Heptanoylglycine	$M+NH_4$	205.1546
HILIC/ +	253.1056	3E-03	0.8	↓ CMO; Combo X Control	Ribose 1-phosphate	M+Na	253.0083
HILIC/ +	115.0622	4E-03	0.8	↑ CMO X Control; Combo	Dihydrouracil	M+H	115.0502
HILIC/ +	161.0923	6E-03	0.8	↑ CMO X Control; Combo	Tryptamine	M+H	161.1073
HILIC/ +	268.1509	1E-02	0.9	↑ CMO X Combo	Neuraminic acid	M+H	268.1026
HILIC/ +	425.2159	1E-02	0.9	↑ Combo X Control; CMO	1b,3a,7b-Trihydroxy-5b-cholanoic acid	M+H	425.2897
HILIC/ +	285.9893	2E-02	0.9	↑ CMO X Combo	Epinephrine sulfate	M+Na	286.0355
HILIC/ +	414.1791[#]	2E-02	0.9	↑ CMO X Control; Combo	Pregnenolone sulfate	$M+NH_4$	414.2309
HILIC/ +	133.1056[#$]	3E-02	0.9	↑ Combo X CMO	Dimethylmalonic acid	M+H	133.04954
HILIC/ +	456.2266	3E-02	0.9	↓ CMO X Control; Combo	Lithocholic acid glycine conjugate	M+Na	456.30843
HILIC/ +	245.0805	3E-02	0.9	↑ Combo X Control; CMO	L-Glutamic acid 5-phosphate	$M+NH_4$	245.0533
					Uridine	M+H	245.0768
HILIC/ -	475.74305[#]	5E-09	1.3E-06	↑ CMO X Control; Combo	Cytidine diphosphate diacylglycerol (16:0/16:0)	M-2H	475.7499

					Inositol-P-ceramide	M-2H	475.8393
HILIC/ -	471.7480	5E-09	1.3E-06	↑ CMO X Control; Combo	Lactosylceramide (d18:1/22:0)	M-2H	471.8485
					cis,cis-3,6-Dodecadienoyl-CoA	M-2H	471.6182
HILIC/ -	477.7417[#]	7E-09	1.7E-06	↑ CMO X Control; Combo	Pentaglutamyl folate	M-2H	477.6478
HILIC/ -	295.6899	1E-08	2.0E-06	↑ CMO X Control; Combo	Ceramide (d18:1/20:0)	M-2H	295.78007
HILIC/ -	298.6857[#]	2E-08	2.9E-06	↑ CMO X Control; Combo	Triglicerides	M-3H	298.6045
HILIC/ -	393.8184[#]	3E-08	3.7E-06	↑ CMO X Control; Combo	Phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine	M-2H	
HILIC/ -	476.7465	4E-08	5.1E-06	↑ CMO X Control; Combo	Cardiolipins	M-3H	476.671
HILIC/ -	391.8210	8E-08	8.5E-06	↑ CMO X Control; Combo	Phosphatidylethanolamines	M-2H	391.80764
					Sphingomyelin (d16:1/24:1(15Z)) Phosphatidylcholines	M-2H	391.81955
HILIC/ -	292.6916	8E-08	8.5E-06	↑ CMO X Control; Combo	Glutaryl-CoA	M-3H	292.70836
					(S)-Hydroxyhexanoyl-CoA	M-3H	292.72049
C18/ +	544.3000[# $]	5E-02	0.5	Combo - Control	Lysophospholipids	M+Na	544.3374

[#] Similar metabolite found in the plasma of pup at weaning. [$] Similar metabolite found in pup after 30 days of weaning.

Appendix G. Annotated plasma metabolites from liquid chromatography-mass spectrometry analysis in HILIC and C18 columns, negative and positive mode, found to separate pup at weaning according to dams treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
HILIC/ +	203.0535	1E-04	0.1	↑ CMO X Control; Combo	D-Glucose or Beta-D-glucose	M+Na	203.0526
					L-Galactose or Beta-D-galactose	M+Na	203.0526
HILIC/ +	414.1791[&]	7E-04	0.2	↑ CMO X Control; Combo	Pregnenolone sulfate	$M+NH_4$	414.2309
HILIC/ +	455.2050	9E-04	0.2	↑ CMO X Control; Combo	3 beta,7 alpha-Dihydroxy-5-cholestenoate	M+Na	455.3132
HILIC/ +	421.9221	2E-03	0.5	↓ CMO; Combo X Control	Monoiodothyronine	M+Na	421.9860
HILIC/ +	299.0900	3E-03	0.5	↓ Combo X Control; CMO	N6-Methyladenosine or 2'-O-Methyladenosine	$M+NH_4$	299.1462
HILIC/ +	168.0774	4E-03	0.5	↑ CMO X Control; Combo	(S)-2-amino-6-oxohexanoate	M+Na	168.0631
					Allysine	M+Na	168.0631
HILIC/ +	245.075[&]	4E-03	0.5	↓ CMO X Control; Combo	L-Glutamic acid 5-phosphate	$M+NH_4$	245.0533
					Uridine	M+H	245.0768
HILIC/ +	126.0220	4E-03	0.5	↓ CMO; Combo X Control	Taurine	M+H	126.0219
HILIC/ +	226.1287	6E-03	0.5	↑ CMO X Control; Combo	Asparaginyl-Alanine etc	M+Na	
HILIC/ +	160.0606[&]	3E-02	0.7	↓ CMO X Control; Combo	1-Methylnicotinamide	M+Na	160.0607
					3-Dehydrocarnitine	M+H	160.0968
HILIC/ +	336.0541	9E-03	0.5	↓ Combo X Control; CMO	5'-phosphoribosyl-a-N-formylglycineamidine	M+Na	336.0567

					Phosphoribosylformylglycinea midine	M+Na	336.0567
HILIC/ +	116.0710	3E-02	0.7	↑ Combo X CMO	Proline	M+H	116.0706
HILIC/ +	166.0867	2E-02	0.6	↓ CMO X Control; Combo	L-Phenylalanine	M+H	166.0863
HILIC/ +	133.0322[&$]	4E-02	0.7	↑ Combo X CMO	Imidazole-4-acetaldehyde	M+Na	133.0372
					Dimethylmalonic acid	M+H	133.0495
					Methylsuccinic acid	M+H	133.0495
					L-Asparagine	M+H	133.0608
HILIC/ +	204.0563	2E-02	0.7	↓ CMO X Control; Combo	L-Tyrosine	M+Na	204.0631
HILIC/ -	377.6035	2E-24	1.8E-21	↑ CMO X Control; Combo	Trihexosylceramide (d18:1/24:0)	M-3H	377.5913
HILIC/ -	373.6079[&]	3E-24	1.8E-21	↑ CMO X Control; Combo	Phosphatidylcholines Phosphatidylethanolamines		
HILIC/ -	375.6058	3E-23	1.3E-20	↑ CMO X Control; Combo	Phosphatidylserines		
HILIC/ -	379.6013	4E-23	1.4E-20	↑ CMO X Control; Combo	Phosphatidylcholines Phosphatidylethanolamines Phosphatidylserines		
HILIC/ -	331.6558	8E-23	2.3E-20	↑ CMO X Control; Combo	Ceramides		
HILIC/ -	371.6101	2E-22	4.3E-20	↑ CMO X Control; Combo	Tetracosanoyl-CoA	M-3H	371.4827
					Phosphatidylethanolamines	M-2H	371.7738
HILIC/ -	333.6534	2E-22	4.5E-20	↑ CMO X Control; Combo	Cytidine diphosphate diacylglycerol (18:1(9Z)/18:2(9Z,12Z))	M-3H	333.5027

HILIC/ -	475.7430[&]	5E-22	9.0E-20	↑ CMO X Control; Combo	Cytidine diphosphate diacylglycerol (16:0/16:0)	M-2H	475.7499
					Inositol-P-ceramide	M-2H	475.8393
HILIC/ -	477.7417[&]	6E-22	9.1E-20	↑ CMO X Control; Combo	Pentaglutamyl folate	M-2H	477.6478
HILIC/ -	471.74808[&]	1E-21	1.3E-19	↑ CMO X Control; Combo	Lactosylceramide (d18:1/22:0)	M-2H	471.8485
					cis,cis-3,6-Dodecadienoyl-CoA	M-2H	471.6182
HILIC/ -	298.6857[&]	6E-21	8.1E-19	↑ CMO X Control; Combo	Triglicerides		
HILIC/ -	260.9807	8E-20	9.0E-18	↑ CMO X Control; Combo	Triglicerides	M-3H	298.6045
HILIC/ -	393.8184[&]	1E-19	1.1E-17	↑ CMO X Control; Combo	Phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine	M-2H	
HILIC/ -	473.7456[&]	5E-19	4.9E-17	↑ CMO X Control; Combo	Flavonoids		
HILIC/ -	429.7960	5E-19	5.0E-17	↑ CMO X Control; Combo	Phosphatidylcholines	M-2H	429.8051
					Lactosylceramide (d18:1/16:0)	M-2H	429.8016
C18/ +	229.2000	9E-03	0.6	↑ CMO X Control; Combo	Isoleucyl-Proline or Leucyl-Proline	M+H	229.1547
C18/ +	424.3000[$]	0.05		↓ CMO X Control; Combo	7-Ketodeoxycholic acid	$M+NH_4$	424.3057
					3-Oxocholic acid	$M+NH_4$	424.3057
					3,7-Dihydroxy-12-oxocholanoic acid	$M+NH_4$	424.3057

C18/ +	544.3000[&$]	0.07		↑ Combo X CMO	Lysophospholipids	M+Na	544.3374
C18/ +	496.3000	0.05		↓ CMO X Control; Combo	Docosa-4,7,10,13,16-pentaenoyl carnitine	M+Na	496.3397
					Clupanodonyl carnitine	M+Na	497.3397
					Lysophospholipid (18:4(6Z,9Z,12Z,15Z)/0:0)	M+Na	496.2435
C18/ +	274.2000	0.2		↑ Combo X CMO	Palmitic acid	$M+NH_4$	274.2741
					L-Thyronine	M+H	274.1074
C18/ -	234.1875	2E-02	0.6	↑ Combo X CMO	Cholesteryl pentanoate	M-2H	234.1989
					Triglycerides	M-2H	234.1731
C18/ -	242.2000	5E-02	0.6	↑ Combo X Control; CMO	Gamma-glutamyl-Proline, Prolyl-Glutamine or Lysyl-Proline	M-H	242.1510
C18/ -	407.2000	0.05		↑ Combo X CMO	9'-Carboxy-gamma-tocotrienol	M+Cl	407.1995
C18/ -	453.2000	0.2		↑ Combo X CMO	Cyclic phosphatidic acid (18:1(11Z)/0:0)	M+Cl	453.2178
					11'-Carboxy-alpha-chromanol	M+Cl	453.2777
C18/ -	431.2718[$]	0.2		↑ Combo X CMO	3 beta,7 alpha-Dihydroxy-5-cholestenoate	M-H	431.3167
					23S,25,26-Trihydroxyvitamin D3	M-H	431.3167
					Ergocalciferol	M+Cl	431.3086

	4a-Methyl-5a-cholesta-8,24-dien-3-one	M+Cl	431.3086

[&] Similar metabolite found in the plasma of dams.

[$] Similar metabolite found in pup after 30 days of weaning.

Appendix H. Annotated plasma metabolites from liquid chromatography-mass spectrometry analysis in HILIC and C18 columns, negative and positive mode, found to separate pup 30 days after weaning according to dams treatment.

Column/Mode	M_{LCMS} [Da]	P-value	FDR	Fisher's LSD	Metabolite candidate	Adduct	Adduct MW
HILIC/ +	259.1520	0.005	0.89	↓ CMO; Combo X Control	Sapropterin	$M+NH_4$	259.1513
					Tetrahydrobiopterin	$M+NH_4$	259.1513
					L-erythro-tetrahydrobiopterin	$M+NH_4$	259.1513
HILIC/ +	355.1739	0.011	0.89	↑ Combo X Control; CMO	Lysyl-Tryptophan or Tryptophyl-Lysine	M+Na	355.1741
HILIC/ +	120.0636	0.016	0.89	↑ CMO X Control; Combo	Succinic acid semialdehyde	$M+NH_4$	120.0655
					Acetoacetic acid	$M+NH_4$	120.0655
					2-Ketobutyric acid	$M+NH_4$	120.0655
					L-Threonine	M+H	120.0655
HILIC/ +	133.0612[#&]	0.016	0.89	↓ CMO X Control; Combo	N-Carbamoylsarcosine	M+H	133.0608
					Dimethylmalonic acid	M+H	133.0495
					Ureidopropionic acid	M+H	133.0608
					2-Hydroxycaproic acid	M+H	133.0859
HILIC/ +	195.9779	0.044	0.89	↑ CMO X Control; Combo	3-Hydroxyhippuric acid	M+H	196.0604
					Dopaquinone	M+H	196.0604
HILIC/ +	709.0751	0.031	0.89	↑ CMO X Control; Combo	Diguanosine diphosphate	M+H	709.1127
HILIC/ +	770.8527	0.007	0.89	↓ Combo X Control; CMO	Ganglioside GD3 (d18:0/20:0)	M+H+Na	770.9209
HILIC/ +	378.9915	0.032	0.89	↑ CMO X Control; Combo	7-Methylguanosine 5'-phosphate	M+H	379.0888
HILIC/ -	546.1740	0.001	0.33	↓ CMO; Combo X Control	2-Methyl-1-hydroxybutyl-ThPP	M+Cl	546.0875

HILIC/ -	363.0318	0.001	0.33	↓ CMO; Combo X Control	Xanthylic acid	M-H	363.0347
HILIC/ -	582.0141	0.002	0.59	↑ CMO X Control; Combo	dTDP-D-glucose or dTDP-D-galactose	$M+NH_4$	582.1096
HILIC/ -	167.0204	0.004	0.63	↑ Combo X Control; CMO	Dimethylmalonic acid	M+Cl	167.0117
					Glutaric acid	M+Cl	167.0117
					Uric acid	M-H	167.0211
HILIC/ -	157.0247	0.005	0.63	↑ Combo X Control; CMO	L-Dihydroorotic acid	M-H	157.0255
HILIC/ -	88.0360	0.005	0.63	↓ Combo X Control; CMO	Sarcosine	M-H	88.0404
					L-Alanine	M-H	88.0404
HILIC/ -	323.0392	0.009	0.63	↑ Combo X Control; CMO	Orotidine	M+Cl	323.0288
					Uridine 5'-monophosphate	M-H	323.0286
HILIC/ -	349.0474	0.018	0.63	↑ CMO X Combo	7-Hydroxyenterolactone	M+Cl	349.0848
HILIC/ -	328.0795	0.022	0.63	↑ Combo X CMO	Cyclic AMP	M-H	328.0452
					Adenosine 2',3'-cyclic phosphate	M-H	328.0452
HILIC/ -	355.1526	0.022	0.63	↑ Combo X CMO	11(R)-HETE	M+Cl	355.2045
HILIC/ -	300.1129	0.031	0.66	↑ Combo X CMO	N-Acetyl-D-galactosamine 1-phosphate	M-H	300.0490
HILIC/ -	480.3111	0.033	0.66	↑ CMO X Combo	Lysophospholipid (18:0/0:0) or LysoPC(15:0)	M-H	480.3096
HILIC/ -	287.9814	0.025	0.65	↓ CMO X Control; Combo	L-Threoneopterin	M+Cl	288.0505
HILIC/ -	306.0585	0.039	0.67	↑ Combo X CMO	Iodotyrosine	M-H	305.9633
					dCMP	M-H	306.0497
					Glutathione	M-H	306.0765
C18/ +	241.2000	0.001	0.08	↓ CMO; Combo X Control	Palmitaldehyde	M+H	241.2526
C18/ +	487.2000	0.002	0.10	↓ CMO; Combo X Control	Glucosylgalactosyl hydroxylysine	M+H	487.2134
					15-Hydroxynorandrostene-3,17-dione	M+Na	487.1939

					glucuronide		
					Estriol-3-glucuronide	M+Na	487.1939
C18/ +	368.2750				20-oxo-leukotriene B4	$M+NH_4$	368.2431
					Eicosapentaenoyl Ethanolamide	M+Na	368.2560
					Monoacylglyceride (18:4(6Z,9Z,12Z,15Z)/0:0/0:0)	$M+NH_4$	368.2795
					5a-Tetrahydrocorticosterone	$M+NH_4$	368.2795
C18/ +	424.3000[#]	0.033	0.27	Control - Combo	7-Ketodeoxycholic acid	$M+NH_4$	424.3057
					3-Oxocholic acid	$M+NH_4$	424.3057
					3,7-Dihydroxy-12-oxocholanoic acid	$M+NH_4$	424.3057
					Linoleyl carnitine	M+H	424.3421
C18/ +	544.3000[#&]	0.019	0.22	↑ CMO X Control; Combo	Lysophospholipids	M+Na	544.3374
C18/ +	374.3000				Tetracosahexaenoic acid	$M+NH_4$	374.3054
					13,14-Dihydro PGF2a	$M+NH_4$	374.2901
					Prostaglandin F1a	$M+NH_4$	374.2901
					Monoacylglyceride (18:1(9Z)/0:0/0:0)	$M+NH_4$	374.3265
					14-HDoHE	$M+NH_4$	374.2690
					Sphingosine 1-phosphate (d16:1-P)	M+Na	374.2067
C18/ +	520.3000				Lysophospholipid (18:2(9Z,12Z))	M+H	520.3398
C18/ -	431.2718[#]	0.005	0.19	↓ CMO X Control; Combo	3 beta,7 alpha-Dihydroxy-5-cholestenoate	M-H	431.3167
					23S,25,26-Trihydroxyvitamin D3	M-H	431.3167
					Ergocalciferol	M+Cl	431.3086
					4a-Methyl-5a-cholesta-8,24-dien-3-one	M+Cl	431.3086

C18/ -	243.1750	0.016	0.19	↓ CMO X Control; Combo	(R)-3-Hydroxy-tetradecanoic acid	M-H	243.1966
					Biotin	M-H	243.0809

[#] Similar metabolite found in the plasma of pup at weaning.

[&] Similar metabolite found in the plasma of dams.

References

1. Beaglehole, R. and R. Bonita, *A scorecard for assessing progress in global public health.* Journal of epidemiology and community health, 2009. **63**(7): p. 507-8.

2. Popkin, B.M., L.S. Adair, and S.W. Ng, *Global nutrition transition and the pandemic of obesity in developing countries.* Nutr Rev, 2012. **70**(1): p. 3-21.

3. Beaglehole, R. and D. Yach, *Globalisation and the prevention and control of non-communicable disease: the neglected chronic diseases of adults.* Lancet, 2003. **362**(9387): p. 903-8.

4. Feachem, R., G. Yamey, and C. Schrade, *A moment of truth for global health.* British Medical Journal, 2010. **340**: p. c2869.

5. Feachem, R.G. and M.A. Koblinsky, *Interventions for the control of diarrhoeal diseases among young children: promotion of breast-feeding.* Bull World Health Organ, 1984. **62**(2): p. 271-91.

6. Kuo, S., A. El Guindy, C.M. Panwala, P.M. Hagan, et al., *Differential appearance of T cell subsets in the large and small intestine of neonatal mice.* Pediatric Research 2001. **49**(4): p. 543-51.

7. Monzavi, R., D. Dreimane, M.E. Geffner, S. Braun, et al., *Improvement in risk factors for metabolic syndrome and insulin resistance in overweight youth who are treated with lifestyle intervention.* Pediatrics, 2006. **117**(6): p. e1111-e1118.

8. Villareal, D.T., B.V. Miller, M. Banks, L. Fontana, et al., *Effect of lifestyle intervention on metabolic coronary heart disease risk factors in obese older adults.* The American journal of clinical nutrition, 2006. **84**(6): p. 1317-1323.

9. Barker, D.J., *Low intelligence. Its relation to length of gestation and rate of foetal growth.* British Journal of Preventive & Social Medicine, 1966. **20**(2): p. 58-66.

10. Barker, D.J., *Childhood causes of adult diseases.* Archives of Disease in Childhood 1988. **63**(7): p. 867-9.

11. Barker, D.J., K. Godfrey, C. Fall, C. Osmond, et al., *Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease.* BMJ: British Medical Journal, 1991: p. 671-675.

12. Barker, D.J., *The fetal origins of diseases of old age.* European Journal of Clinical Nutrition 1992. **46 Suppl 3**: p. S3-9.

13. Gluckman, P.D. and M.A. Hanson, *The developmental origins of the metabolic syndrome.* Trends Endocrinol Metab, 2004. **15**(4): p. 183-7.

14. Hales, C.N. and D.J. Barker, *Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis.* Diabetologia, 1992. **35**(7): p. 595-601.

15. Lucas, A., *Programming by early nutrition in man.* Ciba Found Symp, 1991. **156**: p. 38-50; discussion 50-5.

16. Silveira, P.P., A.K. Portella, M.Z. Goldani, and M.A. Barbieri, *Developmental origins of health and disease (DOHaD).* J Pediatr (Rio J), 2007. **83**(6): p. 494-504.

17. Canani, R.B., M.D. Costanzo, L. Leone, G. Bedogni, et al., *Epigenetic mechanisms elicited by nutrition in early life.* Nutrition Research Reviews 2011. **24**(2): p. 198-205.

18. Sebert, S., D. Sharkey, H. Budge, and M.E. Symonds, *The early programming of metabolic health: is epigenetic setting the missing link?* Am J Clin Nutr, 2011. **94**(6 Suppl): p. 1953S-1958S.

19. Steegers-Theunissen, R.P., S.A. Obermann-Borst, D. Kremer, J. Lindemans, et al., *Periconceptional maternal folic acid use of 400 µg per day is related to increased methylation of the IGF2 gene in the very young child.* Public Library of Science One, 2009. **4**(11): p. e7845.

20. Gluckman, P.D. and M.A. Hanson, *Living with the past: evolution, development, and patterns of disease.* Science, 2004. **305**(5691): p. 1733-6.

21. Gluckman, P.D., W. Cutfield, P. Hofman, and M.A. Hanson, *The fetal, neonatal, and infant environments-the long-term consequences for disease risk.* Early Hum Dev, 2005. **81**(1): p. 51-9.

22. Gluckman, P.D., M.A. Hanson, and T. Buklijas, *A conceptual framework for the developmental origins of health and disease.* Journal of Developmental Origins of Health and Disease, 2010. **1**(01): p. 6-18.

23. Warner, M.J. and S.E. Ozanne, *Mechanisms involved in the developmental programming of adulthood disease.* Biochem J, 2010. **427**(3): p. 333-47.

24. Thompson, J.D., D.G. Higgins, and T.J. Gibson, *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.* Nucleic Acids Res, 1994. **22**(22): p. 4673-80.

25. Kawai, K., D. Spiegelman, A.H. Shankar, and W.W. Fawzi, *Maternal multiple micronutrient supplementation and pregnancy outcomes in developing countries: meta-analysis and meta-regression.* Bull World Health Organ, 2011. **89**(6): p. 402-411B.

26. Sanz, Y., *Gut microbiota and probiotics in maternal and infant health.* Am J Clin Nutr, 2011. **94 (suppl)**: p. 200S-5S.

27. Fujiwara, R., N. Takemura, J. Watanabe, and K. Sonoyama, *Maternal consumption of fructo-oligosaccharide diminishes the severity of skin inflammation in offspring of NC/Nga mice.* British Journal of Nutrition 2010. **103**(4): p. 530-8.

28. Backhed, F., *Programming of host metabolism by the gut microbiota.* Annals of Nutrition and Metabolism 2011. **58 Suppl 2**: p. 44-52.

29. Kaplan, J.L. and W.A. Walker, *Early gut colonization and subsequent obesity risk.* Current Opinion in Clinical Nutrition and Metabolic Care 2012. **15**(3): p. 278-84.

30. Jedrychowski, W., F. Perera, U. Maugeri, E. Mroz, et al., *Wheezing and asthma may be enhanced by broad spectrum antibiotics used in early childhood. Concept and results of a pharmacoepidemiology study.* Journal of physiology and pharmacology: an official journal of the Polish Physiological Society, 2011. **62**(2): p. 189.

31. Bedford Russell, A.R. and S.H. Murch, *Could peripartum antibiotics have delayed health consequences for the infant?* British Journal of Obstetrics and Gynaecology 2006. **113**(7): p. 758-65.

32. Neu, J., *Gastrointestinal development and meeting the nutritional needs of premature infants.* Am J Clin Nutr, 2007. **85**(2): p. 629S-634S.

33. Montgomery, R.K., A.E. Mulberg, and R.J. Grand, *Development of the human gastrointestinal tract: twenty years of progress.* Gastroenterology, 1999. **116**(3): p. 702-31.

34. Cheng, H., *Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. IV. Paneth cells.* Am J Anat, 1974. **141**(4): p. 521-35.

35. Louis, N.A. and P.W. Lin, *The intestinal immune barrier.* NeoReviews, 2009. **10**(4): p. e180-e190.

36. Pácha, J., *Development of intestinal transport function in mammals.* Physiological reviews, 2000. **80**(4): p. 1633-1667.

37. Rumbo, M. and E. Schiffrin, *Ontogeny of intestinal epithelium immune functions: developmental and environmental regulation.* Cell Mol Life Sci, 2005. **62**(12): p. 1288-1296.

38. Cummins, A.G. and F.M. Thompson, *Effect of breast milk and weaning on epithelial growth of the small intestine in humans.* Gut, 2002. **51**(5): p. 748-54.

39. Gershon, M.D., A. Chalazonitis, and T.P. Rothman, *From neural crest to bowel: development of the enteric nervous system.* J Neurobiol, 1993. **24**(2): p. 199-214.

40. Burns, A.J. and V. Pachnis, *Development of the enteric nervous system: bringing together cells, signals and genes.* Neurogastroenterol Motility, 2009. **21**(2): p. 100-2.

41. Furness, J. and M. Costa, *The adrenergic innervation of the gastrointestinal tract*1974: Springer.

42. Renz, H., P. Brandtzaeg, and M. Hornef, *The impact of perinatal immune development on mucosal homeostasis and chronic inflammation.* Nat Rev Immunol, 2012. **12**(1): p. 9-23.

43. Furness, J.B., *The enteric nervous system and neurogastroenterology.* Nat Rev Gastroenterol Hepatol, 2012. **9**(5): p. 286-94.

44. Le Douarin, N.M. and C. Kalcheim, *The Neural Crest*1999: Cambridge: Cambridge University Press.

45. Wallace, A.S. and A.J. Burns, *Development of the enteric nervous system, smooth muscle and interstitial cells of Cajal in the human gastrointestinal tract.* Cell Tissue Res, 2005. **319**(3): p. 367-82.

46. Al-Shboul, O.A., *The importance of interstitial cells of cajal in the gastrointestinal tract.* Saudi journal of gastroenterology, 2013. **19**(1): p. 3-15.

47. Ward, S.M., E.A. Beckett, X. Wang, F. Baker, et al., *Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons.* The Journal of Neuroscience, 2000. **20**(4): p. 1393-1403.

48. Young, H.M., D. Ciampoli, B.R. Southwell, and D.F. Newgreen, *Origin of interstitial cells of Cajal in the mouse intestine.* Dev Biol, 1996. **180**(1): p. 97-107.

49. Ward, S.M., S.C. Harney, J.R. Bayguinov, G.J. McLaren, et al., *Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the tunica muscularis.* J Physiol, 1997. **505 (Pt 1)**: p. 241-58.

50. McLain, C.R., Jr., *Amniography Studies of the Gastrointestinal Motility of the Human Fetus.* Am J Obstet Gynecol, 1963. **86**: p. 1079-87.

51. Berseth, C.L., *Motor function in the stomach and small intestine in the neonate.* NeoReviews, 2006. **7**: p. E28-33.

52. Van Haver, E., L. De Vooght, M. Oste, P.T. Sangild, et al., *Postnatal and diet-dependent increases in enteric glial cells and VIP-containing neurones in preterm pigs.* Neurogastroenterology & Motility, 2008. **20**(9): p. 1070-1079.

53. Martin, C.R. and W.A. Walker, *Intestinal immune defences and the inflammatory response in necrotising enterocolitis.* Semin Fetal Neonatal Med, 2006. **11**(5): p. 369-77.

54. Maheshwari, A., *Role of cytokines in human intestinal villous development.* Clin Perinatol, 2004. **31**(1): p. 143-55.

55. Bland, P.W. and D.M. Kambarage, *Antigen handling by the epithelium and lamina propria macrophages.* Gastroenterology Clinics of North America 1991. **20**(3): p. 577-96.

56. Shao, L., O. Kamalu, and L. Mayer, *Non-classical MHC class I molecules on intestinal epithelial cells: mediators of mucosal crosstalk.* Immunol Rev, 2005. **206**: p. 160-76.

57. Rumbo, M. and E.J. Schiffrin, *Ontogeny of intestinal epithelium immune functions: developmental and environmental regulation.* Cell Mol Life Sci, 2005. **62**(12): p. 1288-96.

58. Claud, E.C., L. Lu, P.M. Anton, T. Savidge, et al., *Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(19): p. 7404-8.

59. Lotz, M., D. Gutle, S. Walther, S. Menard, et al., *Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells.* J Exp Med, 2006. **203**(4): p. 973-84.

60. Quan, C.P., F. Forestier, and J.P. Bouvet, *Immunoglobulins of the human amniotic fluid.* Am J Reprod Immunol, 1999. **42**(4): p. 219-25.

61. Shah, U., B.L. Dickinson, R.S. Blumberg, N.E. Simister, et al., *Distribution of the IgG Fc receptor, FcRn, in the human fetal intestine.* Pediatric Research 2003. **53**(2): p. 295-301.

62. Mallow, E.B., A. Harris, N. Salzman, J.P. Russell, et al., *Human enteric defensins. Gene structure and developmental expression.* J Biol Chem, 1996. **271**(8): p. 4038-45.

63. Forchielli, M.L. and W.A. Walker, *The role of gut-associated lymphoid tissues and mucosal defence.* British Journal of Nutrition 2005. **93 Suppl 1**: p. S41-8.

64. Guarner, F. and J.R. Malagelada, *Gut flora in health and disease.* Lancet, 2003. **361**(9356): p. 512-9.

65. Schaeffer, C., M. Diab-Assef, M. Plateroti, F. Laurent-Huck, et al., *Cytokine gene expression during postnatal small intestinal development: regulation by glucocorticoids.* Gut, 2000. **47**(2): p. 192-8.

66. Shanker, A., *Is thymus redundant after adulthood?* Immunol Lett, 2004. **91**(2-3): p. 79-86.

67. Thompson, C. and F. Powrie, *Regulatory T cells.* Curr Opin Pharmacol, 2004. **4**(4): p. 408-14.

68. Scheppach, W., *Effects of short chain fatty acids on gut morphology and function.* Gut, 1994. **35**(1 Suppl): p. S35-8.

69. Muncan, V., J. Heijmans, S.D. Krasinski, N.V. Buller, et al., *Blimp1 regulates the transition of neonatal to adult intestinal epithelium.* Nat Commun, 2011. **2**: p. 452.

70. Sabat, P. and C. Veloso, *Ontogenic development of intestinal disaccharidases in the precocial rodent Octodon degus (Octodontidae).* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 2003. **134**(2): p. 393-397.

71. Hooper, L.V., *Bacterial contributions to mammalian gut development.* Trends Microbiol, 2004. **12**(3): p. 129-34.

72. Newburg, D.S., *Oligosaccharides in human milk and bacterial colonization.* Journal of Pediatric Gastroenterology and Nutrition, 2000. **30 Suppl 2**: p. S8-17.

73. De Filippo, C., D. Cavalieri, M. Di Paola, M. Ramazzotti, et al., *Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(33): p. 14691-14696.

74. Fallani, M., S. Amarri, A. Uusijarvi, R. Adam, et al., *Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres.* Microbiology, 2011. **157**(Pt 5): p. 1385-92.

75. Kripke, S.A., A.D. Fox, J.M. Berman, R.G. Settle, et al., *Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids.* JPEN J Parenter Enteral Nutr, 1989. **13**(2): p. 109-16.

76. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.* Gut, 1980. **21**(9): p. 793-8.

77. Saemann, M.D., G.A. Bohmig, C.H. Osterreicher, H. Burtscher, et al., *Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production.* FASEB J, 2000. **14**(15): p. 2380-2.

78. Niers, L.E., H.M. Timmerman, G.T. Rijkers, G.M. van Bleek, et al., *Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines.* Clin Exp Allergy, 2005. **35**(11): p. 1481-9.

79. Inan, M.S., R.J. Rasoulpour, L. Yin, A.K. Hubbard, et al., *The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line.* Gastroenterology, 2000. **118**(4): p. 724-34.

80. Zapolska-Downar, D., A. Siennicka, M. Kaczmarczyk, B. Kolodziej, et al., *Butyrate inhibits cytokine-induced VCAM-1 and ICAM-1 expression in cultured endothelial cells: the role of NF-kappaB and PPARalpha.* J Nutr Biochem, 2004. **15**(4): p. 220-8.

81. Segain, J.P., D. Raingeard de la Bletiere, A. Bourreille, V. Leray, et al., *Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease.* Gut, 2000. **47**(3): p. 397-403.

82. Haller, D., *Intestinal epithelial cell signalling and host-derived negative regulators under chronic inflammation: to be or not to be activated determines the balance towards commensal bacteria.* Neurogastroenterol Motil, 2006. **18**(3): p. 184-99.

83. Netea, M.G., B.J. Kullberg, D.J. de Jong, B. Franke, et al., *NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease.* European Journal of Immunology 2004. **34**(7): p. 2052-9.

84. Strober, W., P.J. Murray, A. Kitani, and T. Watanabe, *Signalling pathways and molecular interactions of NOD1 and NOD2.* Nat Rev Immunol, 2006. **6**(1): p. 9-20.

85. Fasano, A. and T. Shea-Donohue, *Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases.* Nat Clin Pract Gastroenterol Hepatol, 2005. **2**(9): p. 416-22.

86. Kelly, D., S. Conway, and R. Aminov, *Commensal gut bacteria: mechanisms of immune modulation.* Trends Immunol, 2005. **26**(6): p. 326-33.

87. Ferrero, R.L., *Innate immune recognition of the extracellular mucosal pathogen, Helicobacter pylori.* Mol Immunol, 2005. **42**(8): p. 879-85.

88. Rimoldi, M., M. Chieppa, M. Vulcano, P. Allavena, et al., *Intestinal epithelial cells control dendritic cell function.* Ann N Y Acad Sci, 2004. **1029**: p. 66-74.

89. Tsai, Y.T., P.C. Cheng, and T.M. Pan, *The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits.* Appl Microbiol Biotechnol, 2012. **96**(4): p. 853-62.

90. Wang, M., C. Karlsson, C. Olsson, I. Adlerberth, et al., *Reduced diversity in the early fecal microbiota of infants with atopic eczema.* J Allergy Clin Immunol, 2008. **121**(1): p. 129-34.

91. Kalliomaki, M., M.C. Collado, S. Salminen, and E. Isolauri, *Early differences in fecal microbiota composition in children may predict overweight.* Am J Clin Nutr, 2008. **87**(3): p. 534-8.

92. Reinhardt, C., C.S. Reigstad, and F. Backhed, *Intestinal microbiota during infancy and its implications for obesity.* J Pediatr Gastroenterol Nutr, 2009. **48**(3): p. 249-56.

93. Mackie, R.I., A. Sghir, and H.R. Gaskins, *Developmental microbial ecology of the neonatal gastrointestinal tract.* Am J Clin Nutr, 1999. **69**(5): p. 1035S-1045S.

94. Zilberstein, B., A.G. Quintanilha, M.A. Santos, D. Pajecki, et al., *Digestive tract microbiota in healthy volunteers.* Clinics, 2007. **62**(1): p. 47-54.

95. Hayashi, H., R. Takahashi, T. Nishi, M. Sakamoto, et al., *Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism.* Journal of medical microbiology, 2005. **54**(11): p. 1093-1101.

96. Kleerebezem, M. and E.E. Vaughan, *Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity.* Annu Rev Microbiol, 2009. **63**: p. 269-90.

97. McLoughlin, R.M. and K.H. Mills, *Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma.* J Allergy Clin Immunol, 2011. **127**(5): p. 1097-107; quiz 1108-9.

98. Roberts, S.E., C.J. Wotton, J.G. Williams, M. Griffith, et al., *Perinatal and early life risk factors for inflammatory bowel disease.* World J Gastroenterol, 2011. **17**(6): p. 743-9.

99. Cho, I., S. Yamanishi, L. Cox, B.A. Methe, et al., *Antibiotics in early life alter the murine colonic microbiome and adiposity.* Nature, 2012. **488**(7413): p. 621-6.

100. Bager, P., J. Wohlfahrt, and T. Westergaard, *Caesarean delivery and risk of atopy and allergic disease: meta-analyses.* Clinical & Experimental Allergy 2008. **38**(4): p. 634-42.

101. Hooper, L.V., D.R. Littman, and A.J. Macpherson, *Interactions between the microbiota and the immune system.* Science, 2012. **336**(6086): p. 1268-73.

102. Nicholson, J.K., E. Holmes, J. Kinross, R. Burcelin, et al., *Host-gut microbiota metabolic interactions.* Science, 2012. **336**(6086): p. 1262-7.

103. de Vos, W.M. and E.A. de Vos, *Role of the intestinal microbiome in health and disease: from correlation to causation.* Nutrition Reviews 2012. **70 Suppl 1**: p. S45-56.

104. Harris, K., A. Kassis, G. Major, and C.J. Chou, *Is the gut microbiota a new factor contributing to obesity and its metabolic disorders?* J Obes, 2012. **2012**: p. 879151.

105. Stark, P.L. and A. Lee, *The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life.* Journal of Medical Microbiology, 1982. **15**(2): p. 189-203.

106. Jost, T., C. Lacroix, C.P. Braegger, and C. Chassard, *New insights in gut microbiota establishment in healthy breast fed neonates.* PLoS One, 2012. **7**(8): p. e44595.

107. Koenig, J.E., A. Spor, N. Scalfone, A.D. Fricker, et al., *Succession of microbial consortia in the developing infant gut microbiome.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108 Suppl 1**: p. 4578-85.

108. Scholtens, P.A., R. Oozeer, R. Martin, K.B. Amor, et al., *The early settlers: intestinal microbiology in early life.* Annu Rev Food Sci Technol, 2012. **3**: p. 425-47.

109. Pettker, C.M., I.A. Buhimschi, L.K. Magloire, A.K. Sfakianaki, et al., *Value of placental microbial evaluation in diagnosing intra-amniotic infection.* Obstet Gynecol, 2007. **109**(3): p. 739-49.

110. Jimenez, E., M.L. Marin, R. Martin, J.M. Odriozola, et al., *Is meconium from healthy newborns actually sterile?* Res Microbiol, 2008. **159**(3): p. 187-93.

111. Jimenez, E., L. Fernandez, M.L. Marin, R. Martin, et al., *Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section.* Curr Microbiol, 2005. **51**(4): p. 270-4.

112. Hitti, J., D.E. Riley, M.A. Krohn, S.L. Hillier, et al., *Broad-spectrum bacterial rDNA polymerase chain reaction assay for detecting amniotic fluid infection among women in premature labor.* Clin Infect Dis, 1997. **24**(6): p. 1228-32.

113. Satokari, R., T. Gro¨nroos, K. Laitinen, S. Salminen, et al., *Bifidobacterium and Lactobacillus DNA in the human placenta.* Letters in Applied Microbiology, 2009(48): p. 8-12.

114. Steel, J.H., S. Malatos, N. Kennea, A.D. Edwards, et al., *Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor.* Pediatric Research 2005. **57**(3): p. 404-11.

115. Clemente, J.C., L.K. Ursell, L.W. Parfrey, and R. Knight, *The impact of the gut microbiota on human health: an integrative view.* Cell, 2012. **148**(6): p. 1258-70.

116. DiGiulio, D.B., *Diversity of microbes in amniotic fluid.* Seminars in Fetal and Neonatal Medicine, 2012. **17**(1): p. 2-11.

117. Wagner, C.L., S.N. Taylor, and D. Johnson, *Host factors in amniotic fluid and breast milk that contribute to gut maturation.* Clin Rev Allergy Immunol, 2008. **34**(2): p. 191-204.

118. Eberle, F., M. Sirin, M. Binder, and A.H. Dalpke, *Bacterial RNA is recognized by different sets of immunoreceptors.* European Journal of Immunology 2009. **39**(9): p. 2537-47.

119. Obermeier, F., U.G. Strauch, N. Dunger, N. Grunwald, et al., *In vivo CpG DNA/toll-like receptor 9 interaction induces regulatory properties in CD4+CD62L+ T cells which prevent intestinal inflammation in the SCID transfer model of colitis.* Gut, 2005. **54**(10): p. 1428-36.

120. Dalpke, A., J. Frank, M. Peter, and K. Heeg, *Activation of toll-like receptor 9 by DNA from different bacterial species.* Infection and Immunity, 2006. **74**(2): p. 940-6.

121. Kussmann, M. and P.J. Van Bladeren, *The Extended Nutrigenomics - Understanding the Interplay between the Genomes of Food, Gut Microbes, and Human Host.* Front Genet, 2011. **2**: p. 21.

122. Mazmanian, S.K., C.H. Liu, A.O. Tzianabos, and D.L. Kasper, *An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system.* Cell, 2005. **122**(1): p. 107-18.

123. Biasucci, G., M. Rubini, S. Riboni, L. Morelli, et al., *Mode of delivery affects the bacterial community in the newborn gut.* Early Hum Dev, 2010. **86 Suppl 1**: p. 13-5.

124. Dominguez-Bello, M.G., E.K. Costello, M. Contreras, M. Magris, et al., *Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(26): p. 11971-5.

125. Neu, J. and J. Rushing, *Cesarean Versus Vaginal Delivery: Long-term Infant Outcomes and the Hygiene Hypothesis.* Clin Perinatol, 2011. **38**(2): p. 321-31.

126. Watanabe, T., A. Kitani, P.J. Murray, and W. Strober, *NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses.* Nat Immunol, 2004. **5**(8): p. 800-8.

127. Le Huerou-Luron, I., S. Blat, and G. Boudry, *Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects.* Nutr Res Rev, 2010. **23**(1): p. 23-36.

128. Tong, X., L. Yin, R. Washington, D.W. Rosenberg, et al., *The p50-p50 NF-kappaB complex as a stimulus-specific repressor of gene activation.* Mol Cell Biochem, 2004. **265**(1-2): p. 171-83.

129. Harmsen, H.J., A.C. Wildeboer-Veloo, G.C. Raangs, A.A. Wagendorp, et al., *Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods.* J Pediatr Gastroenterol Nutr, 2000. **30**(1): p. 61-7.

130. Agostoni, C., L. Baselli, and M.B. Mazzoni, *Early nutrition patterns and diseases of adulthood: a plausible link?* European Journal of Internal Medicine 2013. **24**(1): p. 5-10.

131. Nobili, V., G. Bedogni, A. Alisi, A. Pietrobattista, et al., *A protective effect of breastfeeding on the progression of non-alcoholic fatty liver disease.* Archives of Disease in Childhood, 2009. **94**(10): p. 801-805.

132. Garrido, D., D.C. Dallas, and D.A. Mills, *Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications.* Microbiology, 2013. **159**(Pt 4): p. 649-64.

133. Watanabe, T., A. Kitani, and W. Strober, *NOD2 regulation of Toll-like receptor responses and the pathogenesis of Crohn's disease.* Gut, 2005. **54**(11): p. 1515-8.

134. Sharma, R., U. Schumacher, V. Ronaasen, and M. Coates, *Rat intestinal mucosal responses to a microbial flora and different diets.* Gut, 1995. **36**(2): p. 209-14.

135. Meslin, J.C., C. Andrieux, T. Sakata, P. Beaumatin, et al., *Effects of galacto-oligosaccharide and bacterial status on mucin distribution in mucosa and on large intestine fermentation in rats.* British Journal of Nutrition 1993. **69**(3): p. 903-12.

136. Bry, L., P.G. Falk, T. Midtvedt, and J.I. Gordon, *A model of host-microbial interactions in an open mammalian ecosystem.* Science, 1996. **273**(5280): p. 1380-3.

137. Dethlefsen, L. and D.A. Relman, *Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108 Suppl 1**: p. 4554-61.

138. Simopoulos, A.P., *n− 3 fatty acids and human health: Defining strategies for public policy.* Lipids, 2001. **36**(1): p. S83-S89.

139. Jensen, R.G., C.J. Lammi-Keefe, R.A. Henderson, V.J. Bush, et al., *Effect of dietary intake of n-6 and n-3 fatty acids on the fatty acid composition of human milk in North America.* The Journal of pediatrics, 1992. **120**(4): p. S87-S92.

140. Qian, J., T. Chen, W. Lu, S. Wu, et al., *Breast milk macro- and micronutrient composition in lactating mothers from suburban and urban Shanghai.* J Paediatr Child Health, 2010. **46**(3): p. 115-20.

141. Zivkovic, A.M., J.B. German, C.B. Lebrilla, and D.A. Mills, *Human milk glycobiome and its impact on the infant gastrointestinal microbiota.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108 Suppl 1**: p. 4653-8.

142. Ramirez-Farias, C., K. Slezak, Z. Fuller, A. Duncan, et al., *Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii.* British Journal of Nutrition 2009. **101**(4): p. 541-50.

143. Totten, S.M., A.M. Zivkovic, S. Wu, U. Ngyuen, et al., *Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers.* J Proteome Res, 2012. **11**(12): p. 6124-33.

144. German, J.B., C.J. Dillard, and R.E. Ward, *Bioactive components in milk.* Current Opinion in Clinical Nutrition and Metabolic Care 2002. **5**(6): p. 653-8.

145. Boehm, G. and B. Stahl, *Oligosaccharides from milk.* Journal of Nutrition, 2007. **137**(3 Suppl 2): p. 847S-9S.

146. Eiwegger, T., B. Stahl, J. Schmitt, G. Boehm, et al., *Human milk–derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro.* Pediatric Research 2004. **56**(4): p. 536-540.

147. Behnsen, J., E. Deriu, M. Sassone-Corsi, and M. Raffatellu, *Probiotics: properties, examples, and specific applications.* Cold Spring Harbor Perspectives in Medicine, 2013. **3**(3): p. a010074.

148. Zivkovic, A.M., J.B. German, C.B. Lebrilla, and D.A. Mills, *Microbes and Health Sackler Colloquium: Human milk glycobiome and its impact on the infant gastrointestinal microbiota.* Proceedings of the National Academy of Sciences of the United States of America, 2010(108 Suppl 1): p. 4653-8.

149. Hosea Blewett, H.J., M.C. Cicalo, C.D. Holland, and C.J. Field, *The immunological components of human milk.* Adv Food Nutr Res, 2008. **54**: p. 45-80.

150. Gnoth, M.J., C. Kunz, E. Kinne-Saffran, and S. Rudloff, *Human milk oligosaccharides are minimally digested in vitro.* J Nutr, 2000. **130**(12): p. 3014-20.

151. Sokol, H., B. Pigneur, L. Watterlot, O. Lakhdari, et al., *Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(43): p. 16731-6.

152. Wu, S., R. Grimm, J.B. German, and C.B. Lebrilla, *Annotation and structural analysis of sialylated human milk oligosaccharides.* J Proteome Res, 2011. **10**(2): p. 856-68.

153. Wu, S., N. Tao, J.B. German, R. Grimm, et al., *Development of an annotated library of neutral human milk oligosaccharides.* J Proteome Res, 2010. **9**(8): p. 4138-51.

154. Bode, L. and E. Jantscher-Krenn, *Structure-function relationships of human milk oligosaccharides.* Advances in Nutrition, 2012. **3**(3): p. 383S-91S.

155. Thurl, S., M. Munzert, J. Henker, G. Boehm, et al., *Variation of human milk oligosaccharides in relation to milk groups and lactational periods.* British Journal of Nutrition 2010. **104**(9): p. 1261-71.

156. Heikkila, M.P. and P.E. Saris, *Inhibition of Staphylococcus aureus by the commensal bacteria of human milk.* J Appl Microbiol, 2003. **95**(3): p. 471-8.

157. Martin, R., E. Jimenez, H. Heilig, L. Fernandez, et al., *Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR.* Appl Environ Microbiol, 2009. **75**(4): p. 965-9.

158. Martin, R., H.G. Heilig, E.G. Zoetendal, E. Jimenez, et al., *Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women.* Res Microbiol, 2007. **158**(1): p. 31-7.

159. Collado, M.C., S. Delgado, A. Maldonado, and J.M. Rodriguez, *Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR.* Letters in Applied Microbiology 2009. **48**(5): p. 523-8.

160. Hunt, K.M., J.A. Foster, L.J. Forney, U.M. Schutte, et al., *Characterization of the diversity and temporal stability of bacterial communities in human milk.* PLoS One, 2011. **6**(6): p. e21313.

161. Martín, R., S. Langa, C. Reviriego, E. Jimenez, et al., *The commensal microflora of human milk: new perspectives for food bacteriotherapy and probiotics.* Trends in Food Science & Technology, 2004. **15**(3-4): p. 121-127.

162. Cabrera-Rubio, R., M.C. Collado, K. Laitinen, S. Salminen, et al., *The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery.* The American Journal of Clinical Nutrition, 2012. **96**(3): p. 544-51.

163. Solis, G., C.G. de Los Reyes-Gavilan, N. Fernandez, A. Margolles, et al., *Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut.* Anaerobe, 2010. **16**(3): p. 307-310.

164. Martin, V., A. Maldonado-Barragan, L. Moles, M. Rodriguez-Banos, et al., *Sharing of bacterial strains between breast milk and infant feces.* J Hum Lact, 2012. **28**(1): p. 36-44.

165. Gursel, I., M. Gursel, H. Yamada, K.J. Ishii, et al., *Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation.* J Immunol, 2003. **171**(3): p. 1393-400.

166. Bouladoux, N., J.A. Hall, J.R. Grainger, L.M. dos Santos, et al., *Regulatory role of suppressive motifs from commensal DNA.* Mucosal Immunology 2012. **5**(6): p. 623-34.

167. Lin, P.W., T.R. Nasr, and B.J. Stoll, *Necrotizing enterocolitis: recent scientific advances in pathophysiology and prevention.* Semin Perinatol, 2008. **32**(2): p. 70-82.

168. Stagg, A.J., A.L. Hart, S.C. Knight, and M.A. Kamm, *The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria.* Gut, 2003. **52**(10): p. 1522-9.

169. Perez, P.F., J. Dore, M. Leclerc, F. Levenez, et al., *Bacterial imprinting of the neonatal immune system: lessons from maternal cells?* Pediatrics, 2007. **119**(3): p. e724-32.

170. Labeta, M.O., K. Vidal, J.E. Nores, M. Arias, et al., *Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14.* J Exp Med, 2000. **191**(10): p. 1807-12.

171. Dasanayake, A.P., Y. Li, H. Wiener, J.D. Ruby, et al., *Salivary Actinomyces naeslundii genospecies 2 and Lactobacillus casei levels predict pregnancy outcomes.* Journal of Periodontology 2005. **76**(2): p. 171-7.

172. Bearfield, C., E. Davenport, V. Sivapathasundaram, and R. Allaker, *Possible association between amniotic fluid micro-organism infection and microflora in the mouth.* British Journal of Obstetrics and Gynaecology 2002. **109**: p. 527-33.

173. Donnet-Hughes, A., P.F. Perez, J. Dore, M. Leclerc, et al., *Potential role of the intestinal microbiota of the mother in neonatal immune education.* Proceedings Of The Nutrition Society 2010. **69**(3): p. 407-15.

174. Farache, J., I. Koren, I. Milo, I. Gurevich, et al., *Luminal Bacteria Recruit CD103< sup>+</sup> Dendritic Cells into the Intestinal Epithelium to Sample Bacterial Antigens for Presentation.* Immunity, 2013. **38**(3): p. 581-595.

175. Macpherson, A.J. and T. Uhr, *Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria.* Science, 2004. **303**(5664): p. 1662-5.

176. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, et al., *Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.* Nat Immunol, 2001. **2**(4): p. 361-7.

177. Nagl, M., L. Kacani, B. Mullauer, E.M. Lemberger, et al., *Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells.* Clin Diagn Lab Immunol, 2002. **9**(6): p. 1165-8.

178. Uhlig, H.H. and F. Powrie, *Dendritic cells and the intestinal bacterial flora: a role for localized mucosal immune responses.* J Clin Invest, 2003. **112**(5): p. 648-51.

179. Nassif, X., S. Bourdoulous, E. Eugene, and P.O. Couraud, *How do extracellular pathogens cross the blood-brain barrier?* Trends Microbiol, 2002. **10**(5): p. 227-32.

180. Mor, G., I. Cardenas, V. Abrahams, and S. Guller, *Inflammation and pregnancy: the role of the immune system at the implantation site.* Ann N Y Acad Sci, 2011. **1221**(1): p. 80-7.

181. Jamieson, D.J., R.N. Theiler, and S.A. Rasmussen, *Emerging infections and pregnancy.* Emerg Infect Dis, 2006. **12**(11): p. 1638-43.

182. Wloch, S., A. Palasz, and M. Kaminski, *Active and passive transport of drugs in the human placenta.* Ginekol Pol, 2009. **80**(10): p. 772-7.

183. Picciano, M.F., *Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements.* J Nutr, 2003. **133**(6): p. 1997S-2002S.

184. Zeisel, S.H., *Is maternal diet supplementation beneficial? Optimal development of infant depends on mother's diet.* Am J Clin Nutr, 2009. **89**(2): p. 685S-7S.

185. Zerfu, T.A. and H.T. Ayele, *Micronutrients and pregnancy; effect of supplementation on pregnancy and pregnancy outcomes: a systematic review.* Nutr J, 2013. **12**: p. 20.

186. Buppasiri, P., P. Lumbiganon, J. Thinkhamrop, C. Ngamjarus, et al., *Calcium supplementation (other than for preventing or treating hypertension) for improving pregnancy and infant outcomes.* Cochrane Database of Systematic Reviews 2011(10): p. CD007079.

187. Hofmeyr, G.J., T.A. Lawrie, A.N. Atallah, and L. Duley, *Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems.* Cochrane Database of Systematic Reviews 2010(8): p. CD001059.

188. Belizán, J.M., J. Villar, E. Bergel, A.d. Pino, et al., *Long term effect of calcium supplementation during pregnancy on the blood pressure of offspring: follow up of a randomised controlled trial.* British Medical Journal, 1997. **315**(7103): p. 281-285.

189. Santacruz, A., M.C. Collado, L. Garcia-Valdes, M.T. Segura, et al., *Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women.* British Journal of Nutrition 2010. **104**(1): p. 83-92.

190. Africa, C.W., *Oral colonization of Gram-negative anaerobes as a risk factor for preterm delivery.* Virulence, 2011. **6**: p. 498 - 508.

191. Durand, R., E.L. Gunselman, J.S. Hodges, A.J. Diangelis, et al., *A pilot study of the association between cariogenic oral bacteria and preterm birth.* Oral Dis, 2009. **15**(6): p. 400-6.

192. Africa, C.W., *Oral colonization of Gram-negative anaerobes as a risk factor for preterm delivery.* Virulence, 2011. **2**(6): p. 498-508.

193. Fak, F., S. Ahrne, G. Molin, B. Jeppsson, et al., *Microbial manipulation of the rat dam changes bacterial colonization and alters properties of the gut in her offspring.* American Journal of Physiology-Gastrointestinal and Liver Physiology 2008. **294**(1): p. G148-54.

194. Cotten, C.M., S. Taylor, B. Stoll, R.N. Goldberg, et al., *Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants.* Pediatrics, 2009. **123**(1): p. 58-66.

195. Wang, Y., J.D. Hoenig, K.J. Malin, S. Qamar, et al., *16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis.* ISME J, 2009. **3**(8): p. 944-54.

196. Rowe, J., M. Kusel, B.J. Holt, D. Suriyaarachchi, et al., *Prenatal versus postnatal sensitization to environmental allergens in a high-risk birth cohort.* J Allergy Clin Immunol, 2007. **119**(5): p. 1164-73.

197. Gronlund, M.M., M. Gueimonde, K. Laitinen, G. Kociubinski, et al., *Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the Bifidobacterium microbiota in infants at risk of allergic disease.* Clin Exp Allergy, 2007. **37**(12): p. 1764-72.

198. Dong, P., Y. Yang, and W.P. Wang, *The role of intestinal bifidobacteria on immune system development in young rats.* Early Human Development 2010. **86**(1): p. 51-8.

199. Ogra, P.L. and R.C. Welliver, Sr., *Effects of early environment on mucosal immunologic homeostasis, subsequent immune responses and disease outcome.* Nestle Nutr Workshop Ser Pediatr Program, 2008. **61**: p. 145-81.

200. Round, J.L. and S.K. Mazmanian, *Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(27): p. 12204-9.

201. Herberth, G., D. Hinz, S. Roder, U. Schlink, et al., *Maternal immune status in pregnancy is related to offspring's immune responses and atopy risk.* Allergy, 2011. **66**: p. 1065-1074.

202. Barile, D., M. Marotta, C. Chu, R. Mehra, et al., *Neutral and acidic oligosaccharides in Holstein-Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry.* Journal of Dairy Science, 2010. **93**(9): p. 3940-9.

203. Prescott, S.L., P. Noakes, B.W. Chow, L. Breckler, et al., *Presymptomatic differences in Toll-like receptor function in infants who have allergy.* J Allergy Clin Immunol, 2008. **122**(2): p. 391-9, 399 e1-5.

204. Marsh, L.M., P.I. Pfefferle, O. Pinkenburg, and H. Renz, *Maternal signals for progeny prevention against allergy and asthma.* Cellular and Molecular Life Sciences, 2011. **68**(11): p. 1851-62.

205. Tannock, G.W., R. Fuller, S.L. Smith, and M.A. Hall, *Plasmid profiling of members of the family Enterobacteriaceae, lactobacilli, and bifidobacteria to study the transmission of bacteria from mother to infant.* J Clin Microbiol, 1990. **28**(6): p. 1225-8.

206. Rauch, M. and S.V. Lynch, *The potential for probiotic manipulation of the gastrointestinal microbiome.* Curr Opin Biotechnol, 2012. **23**(2): p. 192-201.

207. Nishijima, K., K. Shukunami, and F. Kotsuji, *Probiotics affects vaginal flora in pregnant women, suggesting the possibility of preventing preterm labor.* J Clin Gastroenterol, 2005. **39**(5): p. 447-8.

208. Tanaka, A., K. Jung, J. Benyacoub, G. Prioult, et al., *Oral supplementation with Lactobacillus rhamnosus CGMCC 1.3724 prevents development of atopic dermatitis in NC/NgaTnd mice possibly by modulating local production of IFN-gamma.* Exp Dermatol, 2009. **18**(12): p. 1022-7.

209. Gareau, M.G., P.M. Sherman, and W.A. Walker, *Probiotics and the gut microbiota in intestinal health and disease.* Nat Rev Gastroenterol Hepatol, 2010. **7**(9): p. 503-14.

210. Myhre, R., A.L. Brantsaeter, S. Myking, H.K. Gjessing, et al., *Intake of probiotic food and risk of spontaneous preterm delivery.* Am J Clin Nutr, 2011. **93**(1): p. 151-7.

211. Brantsaeter, A.L., R. Myhre, M. Haugen, S. Myking, et al., *Intake of probiotic food and risk of preeclampsia in primiparous women: the Norwegian Mother and Child Cohort Study.* Am J Epidemiol, 2011. **174**(7): p. 807-15.

212. Petr, J. and V. Rada, *Bifidobacteria are obligate inhabitants of the crop of adult laying hens.* J Vet Med B Infect Dis Vet Public Health, 2001. **48**(3): p. 227-33.

213. Kim, J.Y., J.H. Kwon, S.H. Ahn, S.I. Lee, et al., *Effect of probiotic mix (Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial.* Pediatric Allergy and Immunology 2010. **21**(2 Pt 2): p. e386-93.

214. Mohan, R., C. Koebnick, J. Schildt, S. Schmidt, et al., *Effects of Bifidobacterium lactis Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study.* J Clin Microbiol, 2006. **44**(11): p. 4025-31.

215. Lee, S.J., S.J. Cho, and E.A. Park, *Effects of probiotics on enteric flora and feeding tolerance in preterm infants.* Neonatology, 2007. **91**(3): p. 174-9.

216. Petschow, B.W., R. Figueroa, C.L. Harris, L.B. Beck, et al., *Effects of feeding an infant formula containing Lactobacillus GG on the colonization of the intestine: a dose-response study in healthy infants.* J Clin Gastroenterol, 2005. **39**(9): p. 786-90.

217. Tannock, G.W., K. Munro, H.J. Harmsen, G.W. Welling, et al., *Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20.* Appl Environ Microbiol, 2000. **66**(6): p. 2578-88.

218. Gueimonde, M., S. Sakata, M. Kalliomaki, E. Isolauri, et al., *Effect of maternal consumption of lactobacillus GG on transfer and establishment of fecal bifidobacterial microbiota in neonates.* J Pediatr Gastroenterol Nutr, 2006. **42**(2): p. 166-70.

219. Schultz, M., C. Gottl, R.J. Young, P. Iwen, et al., *Administration of oral probiotic bacteria to pregnant women causes temporary infantile colonization.* J Pediatr Gastroenterol Nutr, 2004. **38**(3): p. 293-7.

220. Buddington, R.K., C.H. Williams, B.M. Kostek, K.K. Buddington, et al., *Maternal-to-infant transmission of probiotics: concept validation in mice, rats, and pigs.* Neonatology, 2010. **97**(3): p. 250-6.

221. Blumer, N., S. Sel, S. Virna, C.C. Patrascan, et al., *Perinatal maternal application of Lactobacillus rhamnosus GG suppresses allergic airway inflammation in mouse offspring.* Clinical & Experimental Allergy 2007. **37**(3): p. 348-57.

222. Prescott, S.L., K. Wickens, L. Westcott, W. Jung, et al., *Supplementation with Lactobacillus rhamnosus or Bifidobacterium lactis probiotics in pregnancy increases cord blood interferon-gamma and breast milk transforming growth factor-beta and immunoglobin A detection.* Clin Exp Allergy, 2008. **38**(10): p. 1606-14.

223. Kalliomaki, M., S. Salminen, H. Arvilommi, P. Kero, et al., *Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial.* Lancet, 2001. **357**(9262): p. 1076-9.

224. Kalliomaki, M., S. Salminen, T. Poussa, H. Arvilommi, et al., *Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial.* Lancet, 2003. **361**(9372): p. 1869-71.

225. Kalliomaki, M., S. Salminen, T. Poussa, and E. Isolauri, *Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial.* J Allergy Clin Immunol, 2007. **119**(4): p. 1019-21.

226. Wickens, K., P.N. Black, T.V. Stanley, E. Mitchell, et al., *A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial.* J Allergy Clin Immunol, 2008. **122**(4): p. 788-94.

227. Abrahamsson, T.R., T. Jakobsson, M.F. Bottcher, M. Fredrikson, et al., *Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial.* Journal of Allergy and Clinical Immunology, 2007. **119**(5): p. 1174-80.

228. Lahtinen, S.J., R.J. Boyle, S. Kivivuori, F. Oppedisano, et al., *Prenatal probiotic administration can influence Bifidobacterium microbiota development in infants at high risk of allergy.* J Allergy Clin Immunol, 2009. **123**(2): p. 499-501.

229. Ismail, I.H., F. Oppedisano, S.J. Joseph, R.J. Boyle, et al., *Prenatal administration of Lactobacillus rhamnosus has no effect on the diversity of the early infant gut microbiota.* Pediatr Allergy Immunol, 2012. **23**(3): p. 255-8.

230. Laitinen, K., T. Poussa, and E. Isolauri, *Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial.* The Britsh Journal of Nutrition, 2009. **101**(11): p. 1679-87.

231. Luoto, R., K. Laitinen, M. Nermes, and E. Isolauri, *Impact of maternal probiotic-supplemented dietary counselling on pregnancy outcome and prenatal and postnatal growth: a double-blind, placebo-controlled study.* British Journal of Nutrition 2010. **103**(12): p. 1792-9.

232. Aaltonen, J., T. Ojala, K. Laitinen, T. Poussa, et al., *Impact of maternal diet during pregnancy and breastfeeding on infant metabolic programming: a prospective randomized controlled study.* European Journal of Clinical Nutrition 2010(65): p. 10-19.

233. Kaplas, N., E. Isolauri, A.M. Lampi, T. Ojala, et al., *Dietary counseling and probiotic supplementation during pregnancy modify placental phospholipid fatty acids.* Lipids, 2007. **42**(9): p. 865-70.

234. Calder, P.C., *n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases.* The American Journal of Clinical Nutrition, 2006. **83**(6 Suppl): p. 1505S-1519S.

235. Herrera, E., E. Amusquivar, I. Lopez-Soldado, and H. Ortega, *Maternal lipid metabolism and placental lipid transfer.* Horm Res, 2006. **65 Suppl 3**: p. 59-64.

236. Furuhjelm, C., K. Warstedt, J. Larsson, M. Fredriksson, et al., *Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy.* Acta Paediatr, 2009. **98**(9): p. 1461-7.

237. Rivero-Urgell, M. and A. Santamaria-Orleans, *Oligosaccharides: application in infant food.* Early Human Development, 2001. **65 Suppl**: p. S43-52.

238. Gil-Sanchez, A., H. Demmelmair, J.J. Parrilla, B. Koletzko, et al., *Mechanisms involved in the selective transfer of long chain polyunsaturated Fatty acids to the fetus.* Front Genet, 2011. **2**: p. 57.

239. Guandalini, S., G. Magazzu, A. Chiaro, V. La Balestra, et al., *VSL#3 improves symptoms in children with irritable bowel syndrome: a multicenter, randomized, placebo-controlled, double-blind, crossover study.* J Pediatr Gastroenterol Nutr, 2010. **51**(1): p. 24-30.

240. Neu, J., *Routine probiotics for premature infants: let's be careful!* J Pediatr, 2011. **158**(4): p. 672-4.

241. Boyle, R.J., R.M. Robins-Browne, and M.L. Tang, *Probiotic use in clinical practice: what are the risks?* The American Journal of Clinical Nutrition, 2006. **83**(6): p. 1256-64; quiz 1446-7.

242. Luong, M.L., B. Sareyyupoglu, M. Nguyen, F. Silveira, et al., *Lactobacillus probiotic use in cardiothoracic transplant recipients: a link to invasive Lactobacillus infection?* Transplant Infectious Disease, 2010. **12**(6): p. 561-564.

243. van Reenen, C.A. and L.M. Dicks, *Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: what are the possibilities? A review.* Arch Microbiol, 2011. **193**(3): p. 157-68.

244. Shadid, R., M. Haarman, J. Knol, W. Theis, et al., *Effects of galactooligosaccharide and long-chain fructooligosaccharide supplementation during pregnancy on maternal and neonatal microbiota and immunity--a randomized, double-blind, placebo-controlled study.* Am J Clin Nutr, 2007. **86**(5): p. 1426-37.

245. Roberfroid, M., G.R. Gibson, L. Hoyles, A.L. McCartney, et al., *Prebiotic effects: metabolic and health benefits.* British Journal of Nutrition 2010. **104 Suppl 2**: p. S1-63.

246. Fujiwara, R., J. Watanabe, and K. Sonoyama, *Assessing changes in composition of intestinal microbiota in neonatal BALB/c mice through cluster analysis of molecular markers.* British Journal of Nutrition 2008. **99**(6): p. 1174-7.

247. Desbuards, N., P. Gourbeyre, V. Haure-Mirande, D. Darmaun, et al., *Impact of perinatal prebiotic consumption on gestating mice and their offspring: a preliminary report.* British Journal of Nutrition 2011: p. 1-4.

248. Aufreiter, S., J.H. Kim, and D.L. O'Connor, *Dietary oligosaccharides increase colonic weight and the amount but not concentration of bacterially synthesized folate in the colon of piglets.* Journal of Nutrition, 2011. **141**(3): p. 366-72.

249. Iyer, R. and S.K. Tomar, *Folate: a functional food constituent.* J Food Sci, 2009. **74**(9): p. R114-22.

250. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-9.

251. Bhatia, H., J.L. Hallock, A. Dutta, S. Karkashon, et al., *Short-chain fatty acid-mediated effects on erythropoiesis in primary definitive erythroid cells.* Blood, 2009. **113**(25): p. 6440-8.

252. Fathallah, H., R.S. Weinberg, Y. Galperin, M. Sutton, et al., *Role of epigenetic modifications in normal globin gene regulation and butyrate-mediated induction of fetal hemoglobin.* Blood, 2007. **110**(9): p. 3391-7.

253. Kaneda, R., S. Ueno, Y. Yamashita, Y.L. Choi, et al., *Genome-wide screening for target regions of histone deacetylases in cardiomyocytes.* Circ Res, 2005. **97**(3): p. 210-8.

254. Kukkonen, K., E. Savilahti, T. Haahtela, K. Juntunen-Backman, et al., *Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial.* J Allergy Clin Immunol, 2007. **119**(1): p. 192-8.

255. Kukkonen, K., E. Savilahti, T. Haahtela, K. Juntunen-Backman, et al., *Long-term safety and impact on infection rates of postnatal probiotic and prebiotic (synbiotic) treatment: randomized, double-blind, placebo-controlled trial.* Pediatrics, 2008. **122**(1): p. 8-12.

256. Nikniaz, L., A. Ostadrahimi, R. Mahdavi, M.A. Hejazi, et al., *Effects of Synbiotic Supplementation on Breast Milk Levels of IgA, TGF-beta1, and TGF-beta2.* J Hum Lact, 2013. **29**(4): p. 591-6.

257. Nikniaz, L., R. Mahdavi, A. Ostadrahimi, M.A. Hejazi, et al., *Effects of synbiotic supplementation on total antioxidant capacity of human breastmilk.* Breastfeed Med, 2013. **8**: p. 217-22.

258. Jaeger, R., M. Purpura, J. Kiers, and M. Olson, *Pro-and Prebiotic In Vitro Screening to Identify Potential Best-Match Synbiotics.* Gastroenterology, 2013. **144**(5): p. S-898.

259. Sela, D.A., *Bifidobacterial utilization of human milk oligosaccharides.* Int J Food Microbiol, 2011. **149**(1): p. 58-64.

260. Boehm, G., M. Lidestri, P. Casetta, J. Jelinek, et al., *Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants.* Archives of Disease in Childhood: Fetal & Neonatal 2002. **86**(3): p. F178-81.

261. Fong, B., K. Ma, and P. McJarrow, *Quantification of bovine milk oligosaccharides using liquid chromatography-selected reaction monitoring-mass spectrometry.* Journal of Agricultural and Food Chemistry, 2011. **59**(18): p. 9788-95.

262. Martin-Sosa, S., M.J. Martin, L.A. Garcia-Pardo, and P. Hueso, *Sialyloligosaccharides in human and bovine milk and in infant formulas: variations with the progression of lactation.* Journal of Dairy Science, 2003. **86**(1): p. 52-9.

263. Nakamura, T., H. Kawase, K. Kimura, Y. Watanabe, et al., *Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation.* Journal of Dairy Science, 2003. **86**(4): p. 1315-20.

264. Tao, N., E.J. DePeters, S. Freeman, J.B. German, et al., *Bovine milk glycome.* Journal of Dairy Science, 2008. **91**(10): p. 3768-78.

265. Martinez-Ferez, A., C. Rudloff, A. Guadix, C.A. Henkel, et al., *Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology.* International Dairy Journal, 2006(16): p. 173-181.

266. German, J.B., S.L. Freeman, C.B. Lebrilla, and D.A. Mills, *Human milk oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria.* Nestle Nutrition Workshop Series: Paediatric Programme, 2008. **62**: p. 205-18; discussion 218-22.

267. Mehra, R. and P. Kelly, *Milk Oligosaccharides: Structural and technological aspects.* International Dairy Journal, 2006(16): p. 1334-1340.

268. Daddaoua, A., V. Puerta, P. Requena, A. Martinez-Ferez, et al., *Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis.* Journal of Nutrition, 2006. **136**(3): p. 672-6.

269. Lara-Villoslada, F., E. Debras, A. Nieto, A. Concha, et al., *Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis.* Clinical Nutrition, 2006. **25**(3): p. 477-88.

270. Urashima, T., W.A. Bubb, M. Messer, Y. Tsuji, et al., *Studies of the neutral trisaccharides of goat (Capra hircus) colostrum and of the one- and two-dimensional 1H and 13C NMR spectra of 6'-N-acetylglucosaminyllactose.* Carbohydrate Research, 1994. **262**(2): p. 173-84.

271. Bao, Y., C. Chen, and D.S. Newburg, *Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry.* Analytical Biochemistry, 2013. **433**(1): p. 28-35.

272. Aldredge, D.L., M.R. Geronimo, S. Hua, C.C. Nwosu, et al., *Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures.* Glycobiology, 2013. **23**(6): p. 664-76.

273. Yamashita, K. and A. Kobata, *Oligosaccharides of human milk: V. Isolation and characterization of a new trisaccharide, 6′-galactosyllactose.* Archives of Biochemistry and Biophysics, 1974. **161**(1): p. 164-170.

274. Urashima, T., T. Saito, K. Ohmisya, and K. Shimazaki, *Structural determination of three neutral oligosaccharides in bovine (Holstein-Friesian) colostrum, including the novel trisaccharide; GalNAc alpha 1-3Gal beta 1-4Glc.* Biochimica et Biophysica Acta, 1991. **1073**(1): p. 225-9.

275. Chaturvedi, P. and C.B. Sharma, *Goat milk oligosaccharides: purification and characterization by HPLC and high-field 1H-NMR spectroscopy.* Biochimica et Biophysica Acta, 1988. **967**(1): p. 115-21.

276. Urashima, T., S. Murata, and T. Nakamura, *Structural determination of monosialyl trisaccharides obtained from caprine colostrum.* Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 1997. **116**(4): p. 431-5.

277. Kunz, C. and S. Rudloff, *Biological functions of oligosaccharides in human milk.* Acta Paediatrica, 1993. **82**(11): p. 903-12.

278. Bao, Y., L. Zhu, and D.S. Newburg, *Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis.* Analytical Biochemistry, 2007. **370**(2): p. 206-14.

279. Martinez-Ferez, A., A. Guadix, and E.M. Guadix, *Recovery of caprine milk oligosaccharides with ceramic membranes.* Journal of Membrane Science, 2006. **276**: p. 23-30.

280. Yamashita, K., Y. Tachibana, and A. Kobata, *Oligosaccharides of human milk: structures of three lacto-N-hexaose derivatives with H-haptenic structure.* Archives of Biochemistry and Biophysics, 1977. **182**(2): p. 546-55.

281. Dua, V.K., K. Goso, V.E. Dube, and C.A. Bush, *Characterization of lacto-N-hexaose and two fucosylated derivatives from human milk by high-performance liquid chromatography and proton NMR spectroscopy.* Journal of Chromatography, 1985. **328**: p. 259-69.

282. Sela, D.A., Y. Li, L. Lerno, S. Wu, et al., *An infant-associated bacterial commensal utilizes breast milk sialyloligosaccharides.* J Biol Chem, 2011. **286**(14): p. 11909-18.

283. Fuhrer, A., N. Sprenger, E. Kurakevich, L. Borsig, et al., *Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization.* J Exp Med, 2010. **207**(13): p. 2843-54.

284. Simon, P.M., P.L. Goode, A. Mobasseri, and D. Zopf, *Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides.* Infection and Immunity, 1997. **65**(2): p. 750-7.

285. Schauer, R., *Achievements and challenges of sialic acid research.* Glycoconjugate Journal, 2000. **17**(7-9): p. 485-99.

286. Wang, B. and J. Brand-Miller, *The role and potential of sialic acid in human nutrition.* European Journal of Clinical Nutrition, 2003. **57**(11): p. 1351-69.

287. Silanikove, N., G. Leitnerb, U. Merinc, and C.G. Prosser, *Recent advances in exploiting goat's milk: Quality, safety and production aspects.* Small Ruminant Research, 2010(89): p. 110-124.

288. Yangilar, F., *As a Potentially Functional Food: Goats' Milk and Products.* Journal of Food and Nutrition Research, 2013. **1**(4): p. 68-81.

289. Co-operative, N.Z.D.G. *Our Credentials.* . 2014 [cited 2014 November 21, 2014]; Available from: http://www.dgc.co.nz/.

290. Grant, C., B. Rotherham, S. Sharpe, R. Scragg, et al., *Randomized, double-blind comparison of growth in infants receiving goat milk formula versus cow milk infant formula.* J Paediatr Child Health, 2005. **41**(11): p. 564-8.

291. Zhou, S.J., T. Sullivan, R.A. Gibson, B. Lonnerdal, et al., *Nutritional adequacy of goat milk infant formulas for term infants: a double-blind randomised controlled trial.* British Journal of Nutrition 2014. **111**(9): p. 1641-51.

292. Prosser, C., K. Stelwagen, R. Cummins, P. Guerin, et al., *Reduction in heat-induced gastrointestinal hyperpermeability in rats by bovine colostrum and goat milk powders.* J Appl Physiol 2004. **96**(2): p. 650-4.

293. Park, Y.W. and G.F. Haenlein, *Handbook of milk of non-bovine mammals*2008: John Wiley & Sons.

294. Jenness, R., *Composition of milk*, in *Fundamentals of dairy chemistry*1988, Springer. p. 1-38.

295. Ceballos, L.S., E.R. Morales, and T. Adarve, *Composition of goat and cow milk produced under similar conditions and analysed by identical methodology.* Journal of Food Composition and Analysis, 2009. **22**(322-329).

296. Scano, P., A. Murgia, F.M. Pirisi, and P. Caboni, *A gas chromatography-mass spectrometry-based metabolomic approach for the characterization of goat milk compared with cow milk.* J Dairy Sci, 2014. **97**(10): p. 6057-66.

297. Newburg, D.S., *Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans.* Journal of Animal Science, 2009. **87**(13 Suppl): p. 26-34.

298. Newburg, D.S., G.M. Ruiz-Palacios, and A.L. Morrow, *Human milk glycans protect infants against enteric pathogens.* Annual Review of Nutrition, 2005. **25**: p. 37-58.

299. Le Huerou-Luron, I., S. Blat, and G. Boudry, *Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects.* Nutrition Research Reviews, 2010. **23**(1): p. 23-36.

300. Bertino, E., C. Peila, F. Giuliani, C. Martano, et al., *Metabolism and biological functions of human milk oligosaccharides.* Journal of Biological Regulators & Homeostatic Agents 2012. **26**(3 Suppl): p. 35-8.

301. Albrecht, S., J.A. Lane, K. Marino, K.A. Al Busadah, et al., *A comparative study of free oligosaccharides in the milk of domestic animals.* British Journal of Nutrition 2014. **111**(7): p. 1313-28.

302. Marino, K., J.A. Lane, J.L. Abrahams, W.B. Struwe, et al., *Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography.* Glycobiology, 2011. **21**(10): p. 1317-30.

303. Barile, D., N. Tao, C.B. Lebrilla, J.D. Coisson, et al., *Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides.* International Dairy Journal, 2009. **19**(9): p. 524-530.

304. Oliveira, D.L., R.A. Wilbey, A.S. Grandison, L.C. Duarte, et al., *Separation of Oligosaccharides from caprine milk whey, prior to prebiotic evaluation.* International Dairy Journal, 2012. **24**: p. 102-106.

305. Mehra, R., D. Barile, M. Marotta, C.B. Lebrilla, et al., *Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams.* PLoS One, 2014. **9**(5): p. e96040.

306. 930.15, A.I.-M., *Official Methods of Analysis*, in *In Official Methods of Analysis of AOAC International*2000: Gaithersburg, MD, USA.

307. 968.06, A.I.-M., *Animal feed; Determination of nitrogen content by combustion using Dumas method and calculate protein (crude) content.*, in *In Official Methods of Analysis of AOAC International*2005: Gaithersburg, MD, USA.

308. 954.02, A.I.-M., *Fat (crude) or ether extract in pet food*, in *In Official Methods of Analysis of AOAC International*2005: Gaithersburg, MD, USA.

309. Nollet, L.M. and F. Toldrá, *HPLC Determination of Carbohydrate in Foods*, in *Food analysis by HPLC*2012, CRC Press.

310. Fraser, K., S.J. Harrison, G.A. Lane, D.E. Otter, et al., *HPLC–MS/MS profiling of proanthocyanidins in teas: A comparative study.* Journal of Food Composition and Analysis, 2012. **26**(1): p. 43-51.

311. Inamine, J.M., L.N. Lee, and D.J. LeBlanc, *Molecular and genetic characterization of lactose-metabolic genes of Streptococcus cremoris.* Journal of Bacteriology, 1986. **167**(3): p. 855-62.

312. Martini, M.C., E.C. Lerebours, W.J. Lin, S.K. Harlander, et al., *Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on in vivo lactose digestion.* American Journal of Clinical Nutrition, 1991. **54**(6): p. 1041-6.

313. Hernández-Hernández, O., F. Montañés, A. Clemente, F.J. Moreno, et al., *Characterization of galactooligosaccharides derived from lactulose.* Journal of Chromatography A, 2011. **1218**(42): p. 7691-7696.

314. Rasmussen, S., J.H. Thornley, A.J. Parsons, and S.J. Harrison, *Mathematical model of fructan biosynthesis and polymer length distribution in plants.* Annals of botany, 2013. **111**(6): p. 1219-1231.

315. Benthin, S., J. Nielsen, and J. Villadsen, *Galactose Expulsion during Lactose Metabolism in Lactococcus lactis subsp. cremoris FD1 Due to Dephosphorylation of Intracellular Galactose 6-Phosphate.* Applied and Environmental Microbiology, 1994. **60**(4): p. 1254-9.

316. Cumar, F.A., P.A. Ferchmin, and R. Caputto, *Isolation and Identification of a Lactose Phosphate Ester from Cow Colostrum.* Biochemical and Biophysical Research Communications 1965. **20**: p. 60-2.

317. Casper, J.L., W.L. Wendorff, and D.L. Thomas, *Seasonal changes in protein composition of whey from commercial manufacture of caprine and ovine specialty cheeses.* Journal of Dairy Science, 1998. **81**(12): p. 3117-22.

318. Moreno-Indias, I., N. Castro, A. Morales-delaNuez, D. Sanchez-Macias, et al., *Farm and factory production of goat cheese whey results in distinct chemical composition.* Journal of Dairy Science, 2009. **92**(10): p. 4792-6.

319. Romero-Huelva, M., E. Ramos-Morales, and E. Molina-Alcaide, *Nutrient utilization, ruminal fermentation, microbial abundances, and milk yield and composition in dairy goats fed diets including tomato and cucumber waste fruits.* Journal of Dairy Science, 2012. **95**(10): p. 6015-26.

320. Yang, X., J. Chen, and F. Zhang, *Research on the chemical composition of Saanen goat colostrum.* International Journal of Dairy Technology, 2009. **62**(4): p. 500-504.

321. FDA-USA, *Guidance for Industry - Q3C — Tables and List*, U.S.D.o.H.a.H.S.F.a.D. Administration, Editor 2012, Center for Drug Evaluation and Research Food and Drug Administration: FDA U.S.A.

322. Kavanaugh, D.W., J. O'Callaghan, L.F. Butto, H. Slattery, et al., *Exposure of subsp. to Milk Oligosaccharides Increases Adhesion to Epithelial Cells and Induces a Substantial Transcriptional Response.* PLoS One, 2013. **8**(6): p. e67224.

323. Bode, L., *Recent advances on structure, metabolism, and function of human milk oligosaccharides.* Journal of Nutrition, 2006. **136**(8): p. 2127-30.

324. Kamada, N., G.Y. Chen, N. Inohara, and G. Nunez, *Control of pathogens and pathobionts by the gut microbiota.* Nature Immunology, 2013. **14**(7): p. 685-90.

325. Wong, J.M., R. de Souza, C.W. Kendall, A. Emam, et al., *Colonic health: fermentation and short chain fatty acids.* J Clin Gastroenterol, 2006. **40**(3): p. 235-43.

326. Macpherson, A.J. and N.L. Harris, *Interactions between commensal intestinal bacteria and the immune system.* Nature Reviews Immunology, 2004. **4**(6): p. 478-85.

327. Salminen, S., G.R. Gibson, A.L. McCartney, and E. Isolauri, *Influence of mode of delivery on gut microbiota composition in seven year old children.* Gut, 2004. **53**(9): p. 1388-9.

328. Adlerberth, I. and A.E. Wold, *Establishment of the gut microbiota in Western infants.* Acta Paediatrica, 2009. **98**(2): p. 229-38.

329. Haarman, M. and J. Knol, *Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula.* Appl Environ Microbiol, 2005. **71**(5): p. 2318-24.

330. Roger, L.C., A. Costabile, D.T. Holland, L. Hoyles, et al., *Examination of faecal Bifidobacterium populations in breast- and formula-fed infants during the first 18 months of life.* Microbiology, 2010. **156**(Pt 11): p. 3329-41.

331. Shoaf-Sweeney, K.D. and R.W. Hutkins, *Adherence, anti-adherence, and oligosaccharides preventing pathogens from sticking to the host.* Adv Food Nutr Res, 2009. **55**: p. 101-61.

332. Kitaoka, M., *Bifidobacterial enzymes involved in the metabolism of human milk oligosaccharides.* Advances in Nutrition 2012. **3**(3): p. 422S-9S.

333. Chichlowski, M., J.B. German, C.B. Lebrilla, and D.A. Mills, *The Influence of Milk Oligosaccharides on Microbiota of Infants: Opportunities for Formulas.* Annual Review of Food Science and Technology, 2011. **2**: p. 331-51.

334. Kim, S.-H., d.H. Lee, and D. Meyer, *Supplementation of baby formula with native inulin has a prebiotic effect in formula-fed babies.* Asia Pacific journal of clinical nutrition, 2006. **16**(1): p. 172-177.

335. Mugambi, M.N., A. Musekiwa, M. Lombard, T. Young, et al., *Synbiotics, probiotics or prebiotics in infant formula for full term infants: a systematic review.* Nutr J, 2012. **11**: p. 81.

336. Shoaf, K., G.L. Mulvey, G.D. Armstrong, and R.W. Hutkins, *Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells.* Infect Immun, 2006. **74**(12): p. 6920-8.

337. Silfverdal, S.A., L. Bodin, and P. Olcen, *Protective effect of breastfeeding: an ecologic study of Haemophilus influenzae meningitis and breastfeeding in a Swedish population.* Int J Epidemiol, 1999. **28**(1): p. 152-6.

338. Wostmann, B.S., *The germfree animal in nutritional studies.* Annu Rev Nutr, 1981. **1**: p. 257-79.

339. Oliveira, D.L., A. Costabile, R.A. Wilbey, A.S. Grandison, et al., *In vitro evaluation of the fermentation properties and potential prebiotic activity of caprine cheese whey oligosaccharides in batch culture systems.* Biofactors, 2012. **6**(38): p. 440-9.

340. MacFaddin, J., *Biochemical Tests for Identification of Medical Bacteria*. 2nd ed1980: Williams and Wilkins, Baltimore.

341. Rada, V. and J. Petr, *A new selective medium for the isolation of glucose non-fermenting bifidobacteria from hen caeca.* J Microbiol Methods, 2000. **43**(2): p. 127-32.

342. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning*. Vol. 2. 1989: Cold spring harbor laboratory press New York.

343. Kok, R.G., A. de Waal, F. Schut, G.W. Welling, et al., *Specific detection and analysis of a probiotic Bifidobacterium strain in infant feces.* Appl Environ Microbiol, 1996. **62**(10): p. 3668-72.

344. Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski, et al., *DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.* Nucleic Acids Res, 1990. **18**(22): p. 6531-5.

345. Ventura, M., D. van Sinderen, G.F. Fitzgerald, and R. Zink, *Insights into the taxonomy, genetics and physiology of bifidobacteria.* Antonie Van Leeuwenhoek, 2004. **86**(3): p. 205-23.

346. Vincent, D., D. Roy, F. Mondou, and C. Dery, *Characterization of bifidobacteria by random DNA amplification.* Int J Food Microbiol, 1998. **43**(3): p. 185-93.

347. Reuter, G., G. Klein, and M. Goldberg, *Identification of probiotic cultures in food samples.* Food Research International, 2002. **35**(2): p. 117-124.

348. Samarzija, D., S. Sikora, S. Redzepovic, N. Antunac, et al., *Application of RAPD analysis for identification of Lactococcus lactis subsp. cremoris strains isolated from artisanal cultures.* Microbiol Res, 2002. **157**(1): p. 13-7.

349. Perez, G., E. Cardell, and V. Zarate, *Random amplified polymorphic DNA analysis for differentiation of Leuconostoc mesenteroides subspecies isolated from Tenerife cheese.* Lett Appl Microbiol, 2002. **34**(2): p. 82-5.

350. Cocconcelli, P.S., D. Porro, S. Galandini, and L. Senini, *Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci.* Lett Appl Microbiol, 1995. **21**(6): p. 376-9.

351. Corroler, D., N. Desmasures, and M. Gueguen, *Correlation between polymerase chain reaction analysis of the histidine biosynthesis operon, randomly amplified polymorphic DNA analysis and phenotypic characterization of dairy Lactococcus isolates.* Appl Microbiol Biotechnol, 1999. **51**(1): p. 91-9.

352. Roy, D. and S. Sirois, *Molecular differentiation of Bifidobacterium species with amplified ribosomal DNA restriction analysis and alignment of short regions of the ldh gene.* FEMS Microbiol Lett, 2000. **191**(1): p. 17-24.

353. Barrangou, R., E. Altermann, R. Hutkins, R. Cano, et al., *Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by Lactobacillus acidophilus.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(15): p. 8957-62.

354. Sela, D.A., J. Chapman, A. Adeuya, J.H. Kim, et al., *The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(48): p. 18964-9.

355. Turroni, F., F. Bottacini, E. Foroni, I. Mulder, et al., *Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(45): p. 19514-9.

356. Kiyohara, M., K. Tanigawa, T. Chaiwangsri, T. Katayama, et al., *An exo-alpha-sialidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates.* Glycobiology, 2011. **21**(4): p. 437-47.

357. Hayashi, H., M. Sakamoto, M. Kitahara, and B. Y., *Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP.* Microbiology and Immunology, 2003. **47**: p. 557-570.

358. O'Brien, M. and T. Mitsuoka, *Quantitative fluorometric assay for rapid enzymatic characterization of Bifidobacterium longum and related bifidobacteria.* Microbiol Immunol, 1991. **35**(12): p. 1041-7.

359. Sakata, S., T. Tonooka, S. Ishizeki, M. Takada, et al., *Culture-independent analysis of fecal microbiota in infants, with special reference to Bifidobacterium species.* FEMS Microbiol Lett, 2005. **243**(2): p. 417-23.

360. Turroni, F., C. Peano, D.A. Pass, E. Foroni, et al., *Diversity of bifidobacteria within the infant gut microbiota.* PLoS One, 2012. **7**(5): p. e36957.

361. Matto, J., E. Malinen, M.L. Suihko, M. Alander, et al., *Genetic heterogeneity and functional properties of intestinal bifidobacteria.* J Appl Microbiol, 2004. **97**(3): p. 459-70.

362. Ward, R.E., M. Ninonuevo, D.A. Mills, C.B. Lebrilla, et al., *In vitro fermentation of breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri.* Appl Environ Microbiol, 2006. **72**(6): p. 4497-9.

363. Cummings, J., E. Pomare, W. Branch, C. Naylor, et al., *Short chain fatty acids in human large intestine, portal, hepatic and venous blood.* Gut, 1987. **28**(10): p. 1221-1227.

364. Blaut, M., *Relationship of prebiotics and food to intestinal microflora.* European Journal of Nutrition, 2002. **41**(1): p. i11-i16.

365. Cherbut, C., C. Michel, and G. Lecannu, *The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats.* Journal of Nutrition, 2003. **133**(1): p. 21-27.

366. Dass, N., A. John, A. Bassil, C. Crumbley, et al., *The relationship between the effects of short-chain fatty acids on intestinal motility in vitro and GPR43 receptor activation.* Neurogastroenterology & Motility, 2007. **19**(1): p. 66-74.

367. Xiong, Y., N. Miyamoto, K. Shibata, M.A. Valasek, et al., *Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(4): p. 1045-1050.

368. Bourriaud, C., R. Robins, L. Martin, F. Kozlowski, et al., *Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident.* Journal of Applied Microbiology, 2005. **99**(1): p. 201-212.

369. Gyorgy, P., R.F. Norris, and C.S. Rose, *Bifidus factor. I. A variant of Lactobacillus bifidus requiring a special growth factor.* Arch Biochem Biophys, 1954. **48**(1): p. 193-201.

370. Xiao, J.Z., S. Takahashi, M. Nishimoto, T. Odamaki, et al., *Distribution of in vitro fermentation ability of lacto-N-biose I, a major building block of human milk oligosaccharides, in bifidobacterial strains.* Appl Environ Microbiol, 2010. **76**(1): p. 54-9.

371. Depeint, F., G. Tzortzis, J. Vulevic, K. I'Anson, et al., *Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study.* The American Journal of Clinical Nutrition, 2008. **87**(3): p. 785-91.

372. Goulas, T., A. Goulas, G. Tzortzis, and G.R. Gibson, *Comparative analysis of four beta-galactosidases from Bifidobacterium bifidum NCIMB41171: purification and biochemical characterisation.* Appl Microbiol Biotechnol, 2009. **82**(6): p. 1079-88.

373. Pokusaeva, K., G.F. Fitzgerald, and D. van Sinderen, *Carbohydrate metabolism in Bifidobacteria.* Genes Nutr, 2011. **6**(3): p. 285-306.

374. Asakuma, S., E. Hatakeyama, T. Urashima, E. Yoshida, et al., *Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria.* The Journal of Biological Chemistry, 2011. **286**(40): p. 34583-92.

375. Barboza, M., D.A. Sela, C. Pirim, R.G. Locascio, et al., *Glycoprofiling bifidobacterial consumption of galacto-oligosaccharides by mass spectrometry reveals strain-specific, preferential consumption of glycans.* Applied and Environmental Microbiology, 2009. **75**(23): p. 7319-25.

376. Schell, M.A., M. Karmirantzou, B. Snel, D. Vilanova, et al., *The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(22): p. 14422-7.

377. Ashida, H., A. Miyake, M. Kiyohara, J. Wada, et al., *Two distinct α-L-fucosidases from Bifidobacterium bifidum are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates.* Glycobiology, 2009. **19**(9): p. 1010–1017.

378. Sakurama, H., M. Kiyohara, J. Wada, Y. Honda, et al., *Lacto-N-biosidase Encoded by a Novel Gene of Bifidobacterium longum Subspecies longum Shows Unique Substrate Specificity and Requires a Designated Chaperone for Its Active Expression.* J Biol Chem, 2013. **288**(35): p. 25194-206.

379. LoCascio, R.G., P. Desai, D.A. Sela, B. Weimer, et al., *Broad conservation of milk utilization genes in Bifidobacterium longum subsp. infantis as revealed by comparative genomic hybridization.* Appl Environ Microbiol, 2010. **76**(22): p. 7373-81.

380. Mei, G.-Y., C.M. Carey, S. Tosh, and M. Kostrzynska, *Utilization of different types of dietary fibres by potential probiotics.* Canadian Journal of Microbiology, 2011. **57**(10): p. 857-865.

381. Falony, G., K. Lazidou, A. Verschaeren, S. Weckx, et al., *In vitro kinetic analysis of fermentation of prebiotic inulin-type fructans by Bifidobacterium species reveals four different phenotypes.* Applied and Environmental Microbiology, 2009. **75**(2): p. 454-61.

382. Gibson, G.R. and X. Wang, *Regulatory effects of bifidobacteria on the growth of other colonic bacteria.* J Appl Bacteriol, 1994. **77**(4): p. 412-20.

383. Rossi, M., C. Corradini, A. Amaretti, M. Nicolini, et al., *Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures.* Applied Environmental Microbiology, 2005. **71**(10): p. 6150-6158.

384. Ivanov, II and D.R. Littman, *Modulation of immune homeostasis by commensal bacteria.* Curr Opin Microbiol, 2011. **14**(1): p. 106-14.

385. Asahara, T., K. Nomoto, K. Shimizu, M. Watanuki, et al., *Increased resistance of mice to Salmonella enterica serovar Typhimurium infection by synbiotic administration of Bifidobacteria and transgalactosylated oligosaccharides.* Journal of Applied Microbiology 2001. **91**(6): p. 985-96.

386. Le Leu, R.K., Y. Hu, I.L. Brown, R.J. Woodman, et al., *Synbiotic intervention of Bifidobacterium lactis and resistant starch protects against colorectal cancer development in rats.* Carcinogenesis, 2010. **31**(2): p. 246-51.

387. Xu, J., M.A. Mahowald, R.E. Ley, C.A. Lozupone, et al., *Evolution of symbiotic bacteria in the distal human intestine.* PLoS Biol, 2007. **5**(7): p. e156.

388. Stappenbeck, T.S., L.V. Hooper, and J.I. Gordon, *Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(24): p. 15451-5.

389. Backhed, F., H. Ding, T. Wang, L.V. Hooper, et al., *The gut microbiota as an environmental factor that regulates fat storage.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(44): p. 15718-23.

390. Sjögren, Y.M., S. Tomicic, A. Lundberg, M.F. Böttcher, et al., *Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses.* Clinical & Experimental Allergy, 2009. **39**(12): p. 1842-1851.

391. Rolland-Cachera, M., M. Deheeger, and F. Bellisle, *Increasing prevalence of obesity among 18 year old males in Sweden: evidence for early determinants.* Acta Paediatrica, 1999. **88**(4): p. 365-367.

392. Aagaard, K., J. Ma, K.M. Antony, R. Ganu, et al., *The placenta harbors a unique microbiome.* Science Translational Medicine, 2014. **6**(237): p. 237ra65.

393. Edwards, C.A. and A.M. Parrett, *Intestinal flora during the first months of life: new perspectives.* British Journal of Nutrition 2002. **88 Suppl 1**: p. S11-8.

394. Morelli, L., *Postnatal development of intestinal microflora as influenced by infant nutrition.* J Nutr, 2008. **138**(9): p. 1791S-1795S.

395. Jimenez, E., S. Delgado, L. Fernandez, N. Garcia, et al., *Assessment of the bacterial diversity of human colostrum and screening of staphylococcal and enterococcal populations for potential virulence factors.* Res Microbiol, 2008. **159**(9-10): p. 595-601.

396. Scott, C.L., A.M. Aumeunier, and A.M. Mowat, *Intestinal CD103+ dendritic cells: master regulators of tolerance?* Trends in Immunology, 2011. **32**(9): p. 412-419.

397. West, P., J. Hewitt, and O.M. MURPHY, *The influence of methods of collection and storage on the bacteriology of human milk.* Journal of Applied Bacteriology, 1979. **46**(2): p. 269-277.

398. Duffy, L.C., *Interactions mediating bacterial translocation in the immature intestine.* Journal of Nutrition, 2000. **130**(2S Suppl): p. 432S-436S.

399. Barouei, J., M.C. Adams, and D.M. Hodgson, *Prophylactic role of maternal administration of probiotics in the prevention of irritable bowel syndrome.* Medical Hypotheses 2009. **73**(5): p. 764-7.

400. Lara-Villoslada, F., M. Olivares, S. Sierra, J.M. Rodriguez, et al., *Beneficial effects of probiotic bacteria isolated from breast milk.* British Journal of Nutrition 2007. **98 Suppl 1**: p. S96-100.

401. Kobata, A., *Structures and application of oligosaccharides in human milk.* Proc Jpn Acad Ser B Phys Biol Sci, 2010. **86**(7): p. 731-47.

402. Jones, D.P., Y. Park, and T.R. Ziegler, *Nutritional metabolomics: progress in addressing complexity in diet and health.* Annu Rev Nutr, 2012. **32**: p. 183-202.

403. Shirkey, T.W., R.H. Siggers, B.G. Goldade, J.K. Marshall, et al., *Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig.* Exp Biol Med (Maywood), 2006. **231**(8): p. 1333-45.

404. Goodlad, R.A., B. Ratcliffe, J.P. Fordham, and N.A. Wright, *Does dietary fibre stimulate intestinal epithelial cell proliferation in germ free rats?* Gut, 1989. **30**(6): p. 820-5.

405. Fouhy, F., R.P. Ross, G.F. Fitzgerald, C. Stanton, et al., *Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps.* Gut Microbes, 2012. **3**(3): p. 203-20.

406. Troina, A.A., M.S. Figueiredo, M.C. Passos, A.M. Reis, et al., *Flaxseed bioactive compounds change milk, hormonal and biochemical parameters of dams and offspring during lactation.* Food Chem Toxicol, 2012. **50**(7): p. 2388-96.

407. Nebra, Y. and A. Blanch, *A New Selective Medium forBifidobacterium spp.* Appl Environ Microbiol, 1999. **65**(11): p. 5173-5176.

408. Mueller, S., r.K. Saunie, C. Hanisch, E. Norin, et al., *Differences in Fecal Microbiota in Different European Study Populations in Relation to Age, Gender, and Country: a Cross-Sectional Study .* Appl. Environ. Microbiol., 2006. **72**(2): p. 1027-1033.

409. Ninonuevo, M.R., Y. Park, H. Yin, J. Zhang, et al., *A strategy for annotating the human milk glycome.* J Agric Food Chem, 2006. **54**(20): p. 7471-80.

410. Weitzhandler, M., V. Barreto, C. Pohl, P. Jandik, et al., *CarboPac PA20: a new monosaccharide separator column with electrochemical detection with disposable gold electrodes.* J Biochem Biophys Methods, 2004. **60**(3): p. 309-17.

411. Sommer, F. and F. Backhed, *The gut microbiota--masters of host development and physiology.* Nat Rev Microbiol, 2013. **11**(4): p. 227-38.

412. Coates, M.E., *Gnotobiotic animals in research: their uses and limitations.* Laboratory animals, 1975. **9**(4): p. 275-282.

413. Shimizu, K., Y. Muranaka, R. Fujimura, H. Ishida, et al., *Normalization of reproductive function in germfree mice following bacterial contamination.* Exp Anim, 1998. **47**(3): p. 151-8.

414. Minami, M., T. Oowada, A. Ozaki, T. Yamamoto, et al., *Estrous cycle and vaginal flora in conventionalized and gnotobiotic BALB/c mice.* Bifidus, 1987. **1**(1): p. 25-32.

415. Shinoda, M., H. Tamura, Y. Tajima, K. Maejima, et al., *[The weights of genital organs in germfree and conventional ICR mice administered with hCG, estradiol benzoate, progesterone and testosterone propionate (author's transl)].* Jikken Dobutsu, 1980. **29**(3): p. 345-9.

416. Widdowson, E.M. and R. McCance, *The effect of finite periods of undernutrition at different ages on the composition and subsequent development of the rat.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1963. **158**(972): p. 329-342.

417. Harding, J. and B. Johnston, *Nutrition and fetal growth.* Reproduction, Fertility and Development, 1995. **7**(3): p. 539-547.

418. Thompson, G.R. and P.C. Trexler, *Gastrointestinal structure and function in germ-free or gnotobiotic animals.* Gut, 1971. **12**(3): p. 230-5.

419. Asano, T., *Modification of cecal size in germfree rats by long-term feeding of anion exchange resin.* American Journal of Physiology, 1969. **217**(4): p. 911-918.

420. Wagner, R.D., *Effects of microbiota on GI health: gnotobiotic research.* Adv Exp Med Biol, 2008. **635**: p. 41-56.

421. Tomas, J., L. Wrzosek, N. Bouznad, S. Bouet, et al., *Primocolonization is associated with colonic epithelial maturation during conventionalization.* FASEB J, 2013. **27**(2): p. 645-55.

422. Miniats, O.P. and V.E. Valli, *The gastronintestinal tract of gnotobiotic pigs.* Germfree Research. Biological Effects of Gnotobiotic Environments.1973, New York: Academic Press

423. Fontaine, N., J. Meslin, S. Lory, and C. Andrieux, *Intestinal mucin distribution in the germ-free rat and in the heteroxenic rat harbouring a human bacterial flora: effect of inulin in the diet.* British Journal of Nutrition, 1996. **75**(06): p. 881-892.

424. Gaskins, H., *Immunological aspects of host/microbiota interactions at the intestinal epithelium.* Gastrointestinal microbiology, 1997. **2**: p. 537-587.

425. Barcelo, A., J. Claustre, F. Moro, J.A. Chayvialle, et al., *Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon.* Gut, 2000. **46**(2): p. 218-24.

426. Cabotaje, L.M., F.L. Shinnick, J.M. Lopez-Guisa, and J.A. Marlett, *Mucin secretion in germfree rats fed fiber-free and psyllium diets and bacterial mass and carbohydrate fermentation after colonization.* Applied and Environmental Microbiology, 1994. **60**(4): p. 1302-7.

427. Satchithanandam, S., M. Vargofcak-Apker, R.J. Calvert, A.R. Leeds, et al., *Alteration of gastrointestinal mucin by fiber feeding in rats.* J Nutr, 1990. **120**(10): p. 1179-84.

428. Schmidt-Wittig, U., M.L. Enss, M. Coenen, K. Gartner, et al., *Response of rat colonic mucosa to a high fiber diet.* Ann Nutr Metab, 1996. **40**(6): p. 343-50.

429. Ten Bruggencate, S.J., I.M. Bovee-Oudenhoven, M.L. Lettink-Wissink, M.B. Katan, et al., *Dietary fructooligosaccharides affect intestinal barrier function in healthy men.* J Nutr, 2006. **136**(1): p. 70-4.

430. Tanabe, H., K. Sugiyama, T. Matsuda, S. Kiriyama, et al., *Small intestinal mucins are secreted in proportion to the settling volume in water of dietary indigestible components in rats.* J Nutr, 2005. **135**(10): p. 2431-7.

431. Hoverstad, T. and T. Midtvedt, *Short-chain fatty acids in germfree mice and rats.* J Nutr, 1986. **116**(9): p. 1772-6.

432. Van der Meulen, R., T. Adriany, K. Verbrugghe, and L. De Vuyst, *Kinetic analysis of bifidobacterial metabolism reveals a minor role for succinic acid in the regeneration of NAD^+ through its growth-associated production.* Applied Environmental Microbiology, 2006. **72**(8): p. 5204-5210.

433. den Besten, G., K. van Eunen, A.K. Groen, K. Venema, et al., *The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism.* Journal of Lipid Research, 2013. **54**(9): p. 2325-2340.

434. Palframan, R.J., G.R. Gibson, and R.A. Rastall, *Carbohydrate preferences of Bifidobacterium species isolated from the human gut.* Curr Issues Intest Microbiol, 2003. **4**(2): p. 71-5.

435. de Vries, W. and A.H. Stouthamer, *Fermentation of glucose, lactose, galactose, mannitol, and xylose by bifidobacteria.* Journal of Bacteriology, 1968. **96**(2): p. 472-8.

436. de Vries, W. and A.H. Stouthamer, *Pathway of glucose fermentation in relation to the taxonomy of bifidobacteria.* Journal of Bacteriology, 1967. **93**(2): p. 574-6.

437. Abrams, G.D. and J.E. Bishop, *Effect of the normal microbial flora on gastrointestinal motility.* Proceedings of The Society for Experimental Biology and Medicine 1967. **126**(1): p. 301-4.

438. Yamazaki, S., K. Machii, S. Tsuyuki, H. Momose, et al., *Immunological responses to monoassociated Bifidobacterium longum and their relation to prevention of bacterial invasion.* Immunology, 1985. **56**(1): p. 43-50.

439. Romond, M.B., Z. Haddou, C. Mielcareck, and C. Romond, *Bifidobacteria and human health: regulatory effect of indigenous bifidobacteria on Escherichia coli intestinal colonization.* Anaerobe, 1997. **3**(2-3): p. 131-6.

440. Deitch, E.A., *Bacterial translocation: the influence of dietary variables.* Gut, 1994. **35**(1 Suppl): p. S23-7.

441. Barber, A.E., W.G. Jones, J.P. Minei, T.J. Fahey, et al., *Glutamine or fiber supplementation of a defined formula diet: impact on bacterial translocation, tissue composition, and response to endotoxin.* Journal of Parenteral and Enteral Nutrition, 1990. **14**(4): p. 335-343.

442. El Aidy, S., P. van Baarlen, M. Derrien, D.J. Lindenbergh-Kortleve, et al., *Temporal and spatial interplay of microbiota and intestinal mucosa drive establishment of immune homeostasis in conventionalized mice.* Mucosal immunology, 2012. **5**(5): p. 567-579.

443. Di Mauro, A., J. Neu, G. Riezzo, F. Raimondi, et al., *Gastrointestinal function development and microbiota.* Italian Journal of Pediatrics, 2013. **39**: p. 15.

444. Matamoros, S., C. Gras-Leguen, F. Le Vacon, G. Potel, et al., *Development of intestinal microbiota in infants and its impact on health.* Trends Microbiol, 2013. **21**(4): p. 167-73.

445. Westerbeek, E.A., A. van den Berg, H.N. Lafeber, J. Knol, et al., *The intestinal bacterial colonisation in preterm infants: a review of the literature.* Clin Nutr, 2006. **25**(3): p. 361-8.

446. Vos, A.P., M. Haarman, J.W. van Ginkel, J. Knol, et al., *Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice.* Pediatric Allergy and Immunology 2007. **18**(4): p. 304-12.

447. Boehm, G., M. Lidestri, P. Casetta, J. Jelinek, et al., *Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants.* Arch Dis Child Fetal Neonatal Ed, 2002. **86**(3): p. F178-81.

448. Boehm, G. and G. Moro, *Structural and functional aspects of prebiotics used in infant nutrition.* Journal of Nutrition 2008. **138**(9): p. 1818S-1828S.

449. Nakamura, T. and T. Urashima, *The Milk Oligosaccharides of Domestic Farm Animals.* Trends in Glycoscience and Glycotechnology, 2004. **16**(88): p. 135–142.

450. Lara-Villoslada, F., E. Debras, A. Nieto, A. Concha, et al., *Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis.* Clin Nutr, 2006. **25**(3): p. 477-88.

451. Pan, X.D., F.Q. Chen, T.X. Wu, H.G. Tang, et al., *Prebiotic oligosaccharides change the concentrations of short-chain fatty acids and the microbial population of mouse bowel.* J Zhejiang Univ Sci B, 2009. **10**(4): p. 258-63.

452. Wang, Y., T. Zeng, S.E. Wang, W. Wang, et al., *Fructo-oligosaccharides enhance the mineral absorption and counteract the adverse effects of phytic acid in mice.* Nutrition, 2009. **26**(3): p. 305-11.

453. Reeves, P.G., *Components of the AIN-93 diets as improvements in the AIN-76A diet.* J Nutr, 1997. **127**(5 Suppl): p. 838S-841S.

454. Gangrade, B.K. and C.J. Dominic, *Studies of the male-originating pheromones involved in the Whitten effect and Bruce effect in mice.* Biol Reprod, 1984. **31**(1): p. 89-96.

455. Watts, R., *A simple capillary tube method for the determination of the specific gravity of 25 and 50 micro l quantities of urine.* Journal of clinical pathology, 1971. **24**(7): p. 667-668.

456. 996.11, A.I.-M., *Starch (Total) in Cereal Products. Amyloglucosidase–a-Amylase Method*, in *In Official Methods of Analysis of AOAC International*1996: Gaithersburg, MD, USA.

457. Taylor, K.A., *A modification of the phenol/sulfuric acid assay for total carbohydrates giving more comparable absorbances.* Applied Biochemistry and Biotechnology, 1995. **53**(3): p. 207-214.

458. 2002.05, A.I.-M., *Determination of vitamin D3 (cholecalciferol) in selected foods.*, in *In Official Methods of Analysis of AOAC International*2006: Gaithersburg, MD, USA.

459. 991.43, A.I.-M., *Total, Soluble, and Insoluble Dietary Fibre in Foods*, in *Official Methods of Analysis of AOAC International*2005: Gaithersburg, MD, USA.

460. Jensen, M., R. Cox, and B.B. Jensen, *Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat.* Animal Science, 1995. **61**(02): p. 293-304.

461. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical Biochemistry 1976. **72**: p. 248-54.

462. Fraser, K., G.A. Lane, D.E. Otter, S.J. Harrison, et al., *Non-targeted analysis by LC-MS of major metabolite changes during the oolong tea manufacturing in New Zealand.* Food Chem, 2014. **151**: p. 394-403.

463. Patti, G.J., O. Yanes, and G. Siuzdak, *Innovation: Metabolomics: the apogee of the omics trilogy.* Nature reviews Molecular cell biology, 2012. **13**(4): p. 263-269.

464. Beckonert, O., H.C. Keun, T.M. Ebbels, J. Bundy, et al., *Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts.* Nature protocols, 2007. **2**(11): p. 2692-2703.

465. Theodoridis, G., H.G. Gika, and I.D. Wilson, *LC-MS-based methodology for global metabolite profiling in metabonomics/metabolomics.* TrAC Trends in Analytical Chemistry, 2008. **27**(3): p. 251-260.

466. Pan, G.G. and L.D. Melton, *Analysis of sialyl oligosaccharides by high-performance liquid chromatography-electrospray ionisation-mass spectrometry with differentiation of alpha2-3 and alpha2-6 sialyl linkages.* J Chromatography A, 2005. **1077**(2): p. 136-42.

467. Mann, E., S. Schmitz-Esser, Q. Zebeli, M. Wagner, et al., *Mucosa-associated bacterial microbiome of the gastrointestinal tract of weaned pigs and dynamics linked to dietary calcium-phosphorus.* PLoS One, 2014. **9**(1): p. e86950.

468. Young, W., *The effects of digestion resistant carbohydrates on the colon microbiota and colon tissue transcriptome of weanling rats*, 2011, University of Otago: Thesis, Doctor of Philosophy.

469. Arslanoglu, S., G.E. Moro, J. Schmitt, L. Tandoi, et al., *Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life.* Journal of Nutrition, 2008. **138**(6): p. 1091-5.

470. Gourbeyre, P., N. Desbuards, G. Gremy, S. Le Gall, et al., *Exposure to a galactooligosaccharides/inulin prebiotic mix at different developmental time points differentially modulates immune responses in mice.* J Agric Food Chem, 2012. **60**(48): p. 11942-51.

471. Leforestier, G., A. Blais, F. Blachier, A. Marsset-Baglieri, et al., *Effects of galacto-oligosaccharide ingestion on the mucosa-associated mucins and sucrase activity in the small intestine of mice.* Eur J Nutr, 2009. **48**(8): p. 457-64.

472. Legette, L.L., W. Lee, B.R. Martin, J.A. Story, et al., *Prebiotics enhance magnesium absorption and inulin-based fibers exert chronic effects on calcium utilization in a postmenopausal rodent model.* J Food Sci, 2012. **77**(4): p. H88-94.

473. Maurer, A.D. and R.A. Reimer, *Maternal consumption of high-prebiotic fibre or -protein diets during pregnancy and lactation differentially influences satiety hormones and expression of genes involved in glucose and lipid metabolism in offspring in rats.* British Journal of Nutrition 2011. **105**(3): p. 329-38.

474. Bouillon, R., G. Carmeliet, L. Lieben, M. Watanabe, et al., *Vitamin D and energy homeostasis—of mice and men.* Nature Reviews Endocrinology, 2014. **10**: p. 79-87.

475. Audi, L., G. Marti, C. Esteban, M. Oyarzabal, et al., *VDR gene polymorphism at exon 2 start codon (FokI) may have influenced Type 1 diabetes mellitus susceptibility in two Spanish populations.* Diabetic medicine, 2004. **21**(4): p. 393-394.

476. Hillman, L.S., W. Chow, S.S. Salmons, E. Weaver, et al., *Vitamin D metabolism, mineral homeostasis, and bone mineralization in term infants fed human milk, cow milk-based formula, or soy-based formula.* The Journal of pediatrics, 1988. **112**(6): p. 864-874.

477. Cockburn, F., N. Belton, R. Purvis, M. Giles, et al., *Maternal vitamin D intake and mineral metabolism in mothers and their newborn infants.* British Medical Journal, 1980. **281**(6232): p. 11.

478. Bhat, M., B. Noolu, S.S. Qadri, and A. Ismail, *Vitamin D deficiency decreases adiposity in rats and causes altered expression of uncoupling proteins and steroid receptor coactivator3.* The Journal of Steroid Biochemistry and Molecular Biology, 2014(October): p. 304-312.

479. Sleet, R. and J. Brightwell, *FS-Teratology study in rats*, 1990, Raffinerie Tirlemontoise Internal Report.

480. Henquin, J., *Reproduction toxicity: study on the influence of fructooligosaccharides on the development of foetal and postnatal rat*, 1988, Raffinerie Tirlemontoise Internal Report.

481. Maurer, A.D., Q. Chen, C. McPherson, and R.A. Reimer, *Changes in satiety hormones and expression of genes involved in glucose and lipid metabolism in rats weaned onto diets high in fibre or protein reflect susceptibility to increased fat mass in adulthood.* J Physiol, 2009. **587**(Pt 3): p. 679-91.

482. Wahabi, H.A., A.A. Fayed, R.A. Alzeidan, and A.A. Mandil, *The independent effects of maternal obesity and gestational diabetes on the pregnancy outcomes.* BMC Endocr Disord, 2014. **14**: p. 47.

483. Everard, A., V. Lazarevic, N. Gaïa, M. Johansson, et al., *Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity.* The International Society for Microbial Ecology Journal, 2014(8): p. 2116-30.

484. Mennitti, L.V., L.M. Oyama, J.L. de Oliveira, A.C. Hachul, et al., *Oligofructose supplementation during pregnancy and lactation impairs offspring development and alters the intestinal properties of 21-d-old pups.* Lipids Health Dis, 2014. **13**: p. 26.

485. Hachul, A., L.V. Mennitti, J.L. de Oliveira, M.F. Moreno, et al., *Oligofructose supplementation (10%) during pregnancy and lactation does not change the inflammatory effect of concurrent trans fatty acid ingestion on 21-day-old offspring.* Lipids Health Dis, 2013. **12**(59): p. 12-59.

486. Karlsson, C.L., G. Molin, F. Fak, M.L. Johansson Hagslatt, et al., *Effects on weight gain and gut microbiota in rats given bacterial supplements and a high-energy-dense diet from fetal life through to 6 months of age.* British Journal of Nutrition 2011. **106**(6): p. 887-95.

487. Boumahrou, N., S. Andrei, G. Miranda, C. Henry, et al., *The major protein fraction of mouse milk revisited using proven proteomic tools.* Journal of Physiology and Pharmacology, 2009. **60 Suppl 3**: p. 113-8.

488. Gors, S., M. Kucia, M. Langhammer, P. Junghans, et al., *Technical note: Milk composition in mice--methodological aspects and effects of mouse strain and lactation day.* J Dairy Sci, 2009. **92**(2): p. 632-7.

489. Guilloteau, P., R. Zabielski, H. Hammon, and C. Metges, *Adverse effects of nutritional programming during prenatal and early postnatal life, some aspects of regulation and potential prevention and treatments.* J Physiol Pharmacol, 2009. **60**(Suppl 3): p. 17-35.

490. Robert, C.d., N. Li, R. Caicedo, S. Frost, et al., *Metabolic effects of different protein intakes after short term undernutrition in artificially reared infant rats.* Early Human Development, 2009. **85**(1): p. 41-49.

491. Lucas, A., R. Morley, and T.J. Cole, *Randomised trial of early diet in preterm babies and later intelligence quotient.* British Journal of Nutrition, 1998. **317**(7171): p. 1481-1487.

492. Rolland-Cachera, M., H. Thibault, J. Souberbielle, D. Soulie, et al., *Massive obesity in adolescents: dietary interventions and behaviours associated with weight regain at 2 y follow-up.* International Journal of Obesity, 2004. **28**(4): p. 514-519.

493. Rolland-Cachera, M.-F., M. Deheeger, F. Bellisle, M. Sempe, et al., *Adiposity rebound in children: a simple indicator for predicting obesity.* The American journal of clinical nutrition, 1984. **39**(1): p. 129-135.

494. Hallam, M.C., D. Barile, M. Meyrand, J.B. German, et al., *Maternal high protein or prebiotic fiber diets affect maternal milk composition and gut microbiota in rat dams and their offspring.* Obesity, 2014. **22**(11): p. 2344-51.

495. Blass, E.M. and M.H. Teicher, *Suckling.* Science, 1980. **210**(4465): p. 15-22.

496. Parnell, J.A. and R.A. Reimer, *Effect of prebiotic fibre supplementation on hepatic gene expression and serum lipids: a dose–response study in JCR: LA-cp rats.* British Journal of Nutrition, 2010. **103**(11): p. 1577-1584.

497. van Meer, H., G. Boehm, F. Stellaard, A. Vriesema, et al., *Prebiotic oligosaccharides and the enterohepatic circulation of bile salts in rats.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2008. **294**(2): p. G540-G547.

498. Davis, L.M., I. Martinez, J. Walter, and R. Hutkins, *A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults.* International Journal of Food Microbiology 2010. **144**(2): p. 285-92.

499. Serino, M., E. Luche, S. Gres, A. Baylac, et al., *Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota.* Gut, 2012. **61**(4): p. 543-553.

500. Lam, Y.Y., C.W. Ha, C.R. Campbell, A.J. Mitchell, et al., *Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice.* PLoS One, 2012. **7**(3): p. e34233.

501. Geurts, L., V. Lazarevic, M. Derrien, A. Everard, et al., *Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue.* Frontiers in microbiology, 2011. **2**.

502. Semova, I., J.D. Carten, J. Stombaugh, L.C. Mackey, et al., *Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish.* Cell Host & Microbe, 2012. **12**(3): p. 277-288.

503. Yin, Y.-N., Q.-F. Yu, N. Fu, X.-W. Liu, et al., *Effects of four Bifidobacteria on obesity in high-fat diet induced rats.* World journal of gastroenterology: WJG, 2010. **16**(27): p. 3394.

504. Wall, R., T.M. Marques, O. O'Sullivan, R.P. Ross, et al., *Contrasting effects of Bifidobacterium breve NCIMB 702258 and Bifidobacterium breve DPC 6330 on the composition of murine brain fatty acids and gut microbiota.* Am J Clin Nutr, 2012. **95**(5): p. 1278-1287.

505. Xiao, J., S. Kondo, N. Takahashi, K. Miyaji, et al., *Effects of Milk Products Fermented by< i> Bifidobacterium longum</i> on Blood Lipids in Rats and Healthy Adult Male Volunteers.* J Dairy Sci, 2003. **86**(7): p. 2452-2461.

506. Solga, S.F., G. Buckley, J.M. Clark, A. Horska, et al., *The effect of a probiotic on hepatic steatosis.* Journal of Clinical Gastroenterology, 2008. **42**(10): p. 1117-1119.

507. An, C., T. Kuda, T. Yazaki, H. Takahashi, et al., *FLX pyrosequencing analysis of the effects of the brown-algal fermentable polysaccharides alginate and laminaran on rat cecal microbiotas.* Applied and Environmental Microbiology, 2013. **79**(3): p. 860-866.

508. Miriam, B. and M. Buenviaje, *Quantitative sputum culture and gram stain: pulmonary infection vs. colonization.* Philipp. J. Microbiol. Infect. Dis, 1989. **18**: p. 28-35.

509. Ravussin, Y., O. Koren, A. Spor, C. LeDuc, et al., *Responses of gut microbiota to diet composition and weight loss in lean and obese mice.* Obesity, 2012. **20**(4): p. 738-747.

510. Bishara, J., R. Farah, J. Mograbi, W. Khalaila, et al., *Obesity as a risk factor for Clostridium difficile infection.* Clinical infectious diseases, 2013: p. cit280.

511. Nadal, I., A. Santacruz, A. Marcos, J. Warnberg, et al., *Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents.* International Journal of Obesity, 2008. **33**(7): p. 758-767.

512. Huda-Faujan, N., A.S. Abdulamir, A. Fatimah, O.M. Anas, et al., *The impact of the level of the intestinal short chain Fatty acids in inflammatory bowel disease patients versus healthy subjects.* The Open Biochemistry Journal, 2010. **4**: p. 53.

513. Lifschitz, C.H., M.J. Wolin, and P.J. Reeds, *Characterization of carbohydrate fermentation in feces of formula-fed and breast-fed infants.* Pediatric Research 1990. **27**(2): p. 165-169.

514. Macfarlane, G., G. Gibson, E. Beatty, and J. Cummings, *Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements.* FEMS Microbiology Letters, 1992. **101**(2): p. 81-88.

515. Jensen, B.B., *Possible ways of modifying type and amount of products from microbial fermentation in the gut*, in *8th Symposium on Digestive Physiology in Pigs. Workshop on: Gut Environment. Influence of luminal factors.*2000, Aarhus University: Uppsala, Sweden. p. 12-14.

516. Hansen, C.F., A. Hernández, J. Mansfield, Á. Hidalgo, et al., *A high dietary concentration of inulin is necessary to reduce the incidence of swine dysentery in pigs experimentally challenged with Brachyspira hyodysenteriae.* British Journal of Nutrition, 2011. **106**(10): p. 1506-1513.

517. Roediger, W., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-429.

518. Mortensen, P.B. and M.R. Clausen, *Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease.* Scandinavian Journal of Gastroenterology, 1996. **31**(S216): p. 132-148.

519. Jacobs, L.R. and B.O. Schneeman, *Effects of dietary wheat bran on rat colonic structure and mucosal cell growth.* J Nutr, 1981. **111**(5): p. 798-803.

520. Aghazadeh, A. and M. TahaYazdi, *Effect of butyric acid supplementation and whole wheat inclusion on the performance and carcass traits of broilers.* South African Journal of Animal Science, 2012. **42**(3): p. 241-248.

521. Sakata, T., M. Adachi, M. Hashida, N. Sato, et al., *Effect of n-butyric acid on epithelial cell proliferation of pig colonic mucosa in short-term culture.* DTW. Deutsche Tierarztliche Wochenschrift, 1995. **102**(4): p. 163-164.

522. Freitas, K.d.C., O.M.S. Amancio, and M.B. de Morais, *High-performance inulin and oligofructose prebiotics increase the intestinal absorption of iron in rats with iron deficiency anaemia during the growth phase.* British Journal of Nutrition, 2012. **108**(06): p. 1008-1016.

523. McCullough, J., B. Ratcliffe, N. Mandir, K. Carr, et al., *Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching.* Gut, 1998. **42**(6): p. 799-806.

524. Demigné, C., C. Morand, M.-A. Levrat, C. Besson, et al., *Effect of propionate on fatty acid and cholesterol synthesis and on acetate metabolism in isolated rat hepatocytes.* British Journal of Nutrition, 1995. **74**(02): p. 209-219.

525. Chen, W.-J.L., J.W. Anderson, and D. Jennings, *Propionate may mediate the hypocholesterolemic effects of certain soluble plant fibers in cholesterol-fed rats.* Experimental Biology and Medicine, 1984. **175**(2): p. 215-218.

526. Ruijschop, R.M., A.E. Boelrijk, and M.C. te Giffel, *Satiety effects of a dairy beverage fermented with propionic acid bacteria.* International Dairy Journal, 2008. **18**(9): p. 945-950.

527. Curi, R., J.A. Bond, P.C. Calder, and E.A. Newsholme, *Propionate regulates lymphocyte proliferation and metabolism.* General Pharmacology: The Vascular System, 1993. **24**(3): p. 591-597.

528. Al-Lahham, S.a.H., M.P. Peppelenbosch, H. Roelofsen, R.J. Vonk, et al., *Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2010. **1801**(11): p. 1175-1183.

529. Todesco, T., A.V. Rao, O. Bosello, and D. Jenkins, *Propionate lowers blood glucose and alters lipid metabolism in healthy subjects.* The American Journal of Clinical Nutrition, 1991. **54**(5): p. 860-865.

530. Younes, H., C. Coudray, J. Bellanger, C. Demigné, et al., *Effects of two fermentable carbohydrates (inulin and resistant starch) and their combination on calcium and magnesium balance in rats.* British Journal of Nutrition, 2001. **86**(04): p. 479-485.

531. Abrams, S.A., I.J. Griffin, K.M. Hawthorne, L. Liang, et al., *A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents.* The American Journal of Clinical Nutrition, 2005. **82**(2): p. 471-476.

532. Kruger, M.C., K.E. Brown, G. Collett, L. Layton, et al., *The effect of fructooligosaccharides with various degrees of polymerization on calcium bioavailability in the growing rat.* Experimental Biology and Medicine, 2003. **228**(6): p. 683-688.

533. Chonan, O. and M. Watanuki, *Effect of galactooligosaccharides on calcium absorption in rats.* Journal of Nutritional Science and Vitaminology 1995. **41**(1): p. 95-104.

534. Pérez-Conesa, D., G. López, P. Abellán, and G. Ros, *Bioavailability of calcium, magnesium and phosphorus in rats fed probiotic, prebiotic and synbiotic powder follow-up infant formulas and their effect on physiological and nutritional parameters.* Journal of the Science of Food and Agriculture, 2006. **86**(14): p. 2327-2336.

535. Weaver, C.M., B.R. Martin, C.H. Nakatsu, A.P. Armstrong, et al., *Galactooligosaccharides improve mineral absorption and bone properties in growing rats through gut fermentation.* J Agric Food Chem, 2011. **59**(12): p. 6501-6510.

536. van den Heuvel, E.G., M.H. Schoterman, and T. Muijs, *Transgalactooligosaccharides stimulate calcium absorption in postmenopausal women.* J Nutr, 2000. **130**(12): p. 2938-2942.

537. Takasugi, S., K. Ashida, S. Maruyama, Y. Matsukiyo, et al., *A combination of a dairy product fermented by lactobacilli and galactooligosaccharides shows additive effects on mineral balances in growing rats with hypochlorhydria induced by a proton pump inhibitor.* Biol Trace Elem Res 2013. **153**((1-3)): p. 309-318.

538. Worker, N. and B. Migicovsky, *Effect of vitamin D on the utilization of zinc, cadmium and mercury in the chick.* J Nutr, 1961. **75**(2): p. 222-224.

539. Hardwick, L.L., M.R. Jones, N. Brautbar, and D. Lee, *Magnesium absorption: mechanisms and the influence of vitamin D, calcium and phosphate.* J Nutr, 1991. **121**(1): p. 13-23.

540. Goehring, K.C., A.D. Kennedy, P.A. Prieto, and R.H. Buck, *Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants.* PLoS One, 2014. **9**(7): p. e101692.

541. Gnoth, M.J., S. Rudloff, C. Kunz, and R.K. Kinne, *Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique.* J Biol Chem, 2001. **276**(37): p. 34363-70.

542. Eiwegger, T., B. Stahl, P. Haidl, J. Schmitt, et al., *Prebiotic oligosaccharides: In vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties.* Pediatric Allergy and Immunology 2010. **21**(8): p. 1179-88.

543. Prieto, P.A., *In Vitro and Clinical Experiences with a Human Milk Oligosaccharide, Lacto-N-neoTetraose, and Fructooligosaccharides.* Foods Food Ingredients J., 2005. **210**(11): p. 1018-30.

544. Karasov, W.H. and I.D. Hume, *Vertebrate gastrointestinal system.* Comprehensive Physiology, 1997.

545. Kindel, T., D.M. Lee, and P. Tso, *The mechanism of the formation and secretion of chylomicrons.* Atherosclerosis Supplements, 2010. **11**(1): p. 11-16.

546. Iqbal, J. and M.M. Hussain, *Intestinal lipid absorption.* American Journal of Physiology-Endocrinology and Metabolism, 2009. **296**(6): p. E1183-E1194.

547. García-Gómez, E., B. González-Pedrajo, and I. Camacho-Arroyo, *Role of sex steroid hormones in bacterial-host interactions.* BioMed research international, 2012. **2013**.

548. Kisiela, M., A. Skarka, B. Ebert, and E. Maser, *Hydroxysteroid dehydrogenases (HSDs) in bacteria–A bioinformatic perspective.* The Journal of steroid biochemistry and molecular biology, 2012. **129**(1): p. 31-46.

549. Fukushima, T., A. Kaetsu, H. Lim, and M. Moriyama, *Possible role of 1-methylnicotinamide in the pathogenesis of Parkinson's disease.* Experimental and Toxicologic Pathology, 2002. **53**(6): p. 469-473.

550. Pumpo, R., G. Sarnelli, A. Spinella, G. Budillon, et al., *The metabolism of nicotinamide in human liver cirrhosis: a study on N-methylnicotinamide and 2-pyridone-5-carboxamide production.* The American journal of gastroenterology, 2001. **96**(4): p. 1183-1187.

551. Gębicki, J., A. Sysa-Jędrzejowska, J. Adamus, A. Woźniacka, et al., *1-Methylnicotinamide: a potent anti-inflammatory agent of vitamin origin.* Pol. J. Pharmacol, 2003. **55**: p. 109-112.

552. Wozniacka, A., M. Wieczorkowska, J. Gebicki, and A. Sysa-Jedrzejowska, *Topical application of 1-methylnicotinamide in the treatment of rosacea: a pilot study.* Clinical and experimental dermatology, 2005. **30**(6): p. 632-635.

553. Watała, C., P. Kaźmierczak, M. Dobaczewski, T. Przygodzki, et al., *Anti-diabetic effects of 1-methylnicotinamide (MNA) in streptozocin-induced diabetes in rats.* Pharmacological Reports, 2009. **61**(1): p. 86-98.

554. Bryniarski, K., R. Biedron, A. Jakubowski, S. Chlopicki, et al., *Anti-inflammatory effect of 1-methylnicotinamide in contact hypersensitivity to oxazolone in mice; involvement of prostacyclin.* European Journal of Pharmacology, 2008. **578**(2): p. 332-338.

555. Brzozowski, T., P.C. Konturek, S. Chlopicki, Z. Sliwowski, et al., *Therapeutic potential of 1-methylnicotinamide against acute gastric lesions induced by stress: role of endogenous prostacyclin and sensory nerves.* Journal of Pharmacology and Experimental Therapeutics, 2008. **326**(1): p. 105-116.

556. Chlopicki, S., J. Swies, A. Mogielnicki, W. Buczko, et al., *1-Methylnicotinamide (MNA), a primary metabolite of nicotinamide, exerts anti-thrombotic activity mediated by a cyclooxygenase-2/prostacyclin pathway.* British Journal of Pharmacology, 2007. **152**(2): p. 230-239.

557. KaŸmierczak, P., C. Watala, E.M. S³ominska, R.T. Smoleñski, et al., *1-Methylnicotinamide (MNA) prevents endothelial dysfunction in hypertriglyceridemic and diabetic rats.* Pharmacological Reports, 2008. **60**(127): p. 127-138.

558. Krause, L.J., C.W. Forsberg, and D.L. O'Connor, *Feeding human milk to rats increases Bifidobacterium in the cecum and colon which correlates with enhanced folate status.* J Nutr, 1996. **126**(5): p. 1505-1511.

559. Fenech, M., *The role of folic acid and vitamin B12 in genomic stability of human cells.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2001. **475**(1): p. 57-67.

560. Hibbard, B.M., *THE ROLE OF FOLIC ACID IN PREGNANCY*.* BJOG: An International Journal of Obstetrics & Gynaecology, 1964. **71**(4): p. 529-542.

561. Want, E.J., B.F. Cravatt, and G. Siuzdak, *The expanding role of mass spectrometry in metabolite profiling and characterization.* Chembiochem, 2005. **6**(11): p. 1941-1951.

562. Fuhrer, A., N. Sprenger, E. Kurakevich, L. Borsig, et al., *Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization.* The Journal of Experimental Medicine, 2010. **207**(13): p. 2843-54.

563. Henao-Mejia, J., E. Elinav, C. Jin, L. Hao, et al., *Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity.* Nature, 2012. **482**(7384): p. 179-185.

564. Ridlon, J.M., J.M. Alves, P.B. Hylemon, and J.S. Bajaj, *Cirrhosis, bile acids and gut microbiota.* jhep, 2013. **2013**: p. 003.

565. Delzenne, N.M. and C.M. Williams, *Prebiotics and lipid metabolism.* Current opinion in lipidology, 2002. **13**(1): p. 61-67.

566. Kim, H., G.E. Bartley, S.A. Young, K.H. Seo, et al., *Altered hepatic gene expression profiles associated with improved fatty liver, insulin resistance, and intestinal permeability after hydroxypropyl methylcellulose (HPMC) supplementation in diet-induced obese mice.* J Agric Food Chem, 2013. **61**(26): p. 6404-11.

567. Wiseman, H., *Vitamin D is a membrane antioxidant Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action.* Federation of European Biochemical Societies letters, 1993. **326**(1): p. 285-288.

568. Lönnerdal, B., *Effects of maternal dietary intake on human milk composition.* J Nutr, 1986. **116**(4): p. 499-513.

569. Cavaglieri, C.R., A. Nishiyama, L.C. Fernandes, R. Curi, et al., *Differential effects of short-chain fatty acids on proliferation and production of pro-and anti-inflammatory cytokines by cultured lymphocytes.* Life Science, 2003. **73**(13): p. 1683-1690.

570. Smith, S.A., T. Hulsey, and W. Goodnight, *Effects of obesity on pregnancy.* Journal of Obstetric, Gynecologic, & Neonatal Nursing, 2008. **37**(2): p. 176-184.

571. Stotland, N.E., Y.W. Cheng, L.M. Hopkins, and A.B. Caughey, *Gestational weight gain and adverse neonatal outcome among term infants.* Obstetrics & Gynecology, 2006. **108**(3, Part 1): p. 635-643.