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# Ruminants’ milk in early postnatal brain development in a pig model of the human infant

A thesis presented in partial fulfilment of the requirements for the degree of

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in

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**Ankita Jena**

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*To Mama and Papa*
*for your unconditional*
*love and support*

# Abstract

Given the rapid brain development in the early postnatal period and its sensitivity towards changes in the external environment like nutrition, this period is of utmost importance for determining later life health and well-being. Emerging evidence suggests a link between the triad of early life nutrition, the gut and brain axis and its potential for optimising or retrograding early postnatal brain development. In this context, human breast milk has been most studied. However, whether the development of the brain is responsive towards milk from ruminant species used to make milk formula via modulating gut-derived molecules has not been well understood.

The aim, therefore, of the thesis was to evaluate the effects of milk from bovine, caprine and ovine species on circulatory blood plasma metabolites, brain tissue metabolites and brain tissue gene expression in piglets and establish associations between changes in plasma metabolite profile with neurochemical and molecular features of the brain. The hypothesis was that metabolites in the peripheral circulation would differ between different ruminant milk consumption, influencing brain metabolite and gene expression.

Liquid chromatography-mass spectrometry-based metabolomics was used to profile the plasma, hippocampal, prefrontal cortex, and striatal tissue metabolite relative abundances. NanoString technology was used to evaluate the expression of genes associated with neuro- and cognitive development in the hippocampus, prefrontal cortex, and striatum tissue samples. Multi-omics data integration was used to explore the correlation between plasma and brain lipid profiles.

The results showed that the relative intensity of plasma metabolites differed between bovine, caprine and ovine milk treatments, and lipid metabolites were the predominant features. The bovine group had a higher relative intensity of plasma lipids (e.g., saturated

triglycerides, phosphatidylcholine, sphingomyelin) than the ovine and caprine milk groups, except for unsaturated triglycerides, which had a higher intensity in the ovine milk group. Metabolite profiling of brain regions indicated that the relative intensity of lipid metabolites, mainly phospholipids, changed in response to different milk treatments. Further analysis showed that in the striatum and hippocampus, the relative intensity of phospholipids in the bovine milk group was higher than in the ovine and caprine milk groups. In contrast, the relative intensity of phospholipids in the prefrontal cortex was higher in the ovine milk group than in the other milk groups. Gene expression profiling showed that the expression of genes in the striatum and hippocampus associated with neurotransmission differed between milk treatments. Both increased and decreased gene expressions were observed in response to ovine milk treatment, whereas a similar gene expression pattern was observed between the caprine and ovine milk treatments. No effect of milk treatments was observed on the prefrontal cortex gene expressions. Striatal and hippocampal lipid relative intensities showed a positive association with that of plasma lipids and the prefrontal cortex showed negative associations.

Thus, this PhD research findings suggest that consuming different ruminant milk can impact early postnatal brain development by influencing the peripheral circulatory metabolites in piglets as a model of human infants.

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"यद् भावं तद् भवति"

You become what you believe

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# Abbreviations

| | |
|---|---|
| 5-HT | Serotonin |
| AHR | Aryl hydrocarbon receptor |
| ANOVA | Analysis of variance |
| BBB | Blood-brain barrier |
| BCAA | Branched-chain amino acid |
| BDNF | Brain-derived neurotrophic factor |
| BW | Body weight |
| CER | Ceramide |
| CNS | Central nervous system |
| CV-ANOVA | Analysis of variance of cross-validated residuals |
| DG | Diglycerides |
| DIABLO | Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies |
| EAA | Essential amino acid |
| EEC | Enteroendocrine cells |
| ENS | Enteric nervous system |
| FC | Fold change |
| FDR | False discovery rate |
| GABA | Gamma amino butyric acid |
| GBA | Gut-brain axis |
| GF | Germ-free |
| GLP | Glucagon-like peptide |
| HILIC | Hydrophilic interaction liquid chromatography |
| HMO | Human milk oligosaccharides |
| HPLC | High-performance liquid chromatography |
| IDO | Trp-2,3-dioxygenase and indoleamine-2,3-dioxygenase |
| LC-MS | Liquid chromatography-mass spectrometry |
| LC-PUFA | Long-chain polyunsaturated fatty acids |
| LNAA | Large neutral amino acid |
| LOOCV | Leave-one-out-cross validation |
| LPC | Lysophosphatidylcholine |

| | |
|---|---|
| LPE | Lysophosphatidylethanolamine |
| LPS | Lipopolysaccharides |
| MCT | Microcentrifuge tubes |
| MFGM | Milk fat globule membranes |
| MUFA | Monounsaturated fatty acids |
| PC | Phosphatidylcholine |
| PCA | Principal component analysis |
| PE | Phosphatidylethanolamine |
| PG | Phosphatidylglycerol |
| PLS-DA | Partial least squares discriminant analysis |
| PND | Postnatal day |
| PS | Phosphatidylserine |
| PUFA | Polyunsaturated fatty acids |
| PYY | Peptide YY |
| ROC | Receiver operating characteristic |
| RP-HPLC | Reversed-phase high-performance liquid chromatography |
| SCFA | Short-chain fatty acids |
| SM | Sphingomyelin |
| SPF | Specific pathogen-free |
| TAA | Total amino acid |
| TG | Triglycerides |
| TPH | Tryptophan hydroxylase |
| Trp | Tryptophan |
| VIP | Variable important for projection |
| VN | Vagus nerve |

# Preface

This thesis is written in the form of publishable chapters. The list below presents the publication status of each chapter. The content of the published chapters has been modified to fit with the format of the thesis.

**Chapter 1 Thesis introduction**

This chapter was written as an introductory chapter for this thesis and is not intended for publication.

**Chapter 2 Literature review**

**Jena A**, Montoya CA, Mullaney JA, Dilger RN, Young W, McNabb WC and Roy NC (2020) Gut-Brain Axis in the Early Postnatal Years of Life: A Developmental Perspective. Frontiers in Integrative Neuroscience14:44. doi: 10.3389/fnint.2020.00044

**Chapter 3 Plasma metabolites profiling of peripheral blood in response to ruminants' milk in early postnatal life**

**Jena A**, Montoya CA, Fraser K, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC, & Roy NC

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**Chapter 4 Metabolites profiling of cognitive areas of the brain in response to ruminants' milk in early postnatal life**

**Jena A**, Fraser K, Montoya CA, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC, & Roy NC

To be submitted: Frontiers in Neuroscience

**Chapter 5 Gene expression profiling of cognitive areas of the brain in response to ruminants' milk in early postnatal life**

**Jena A**, Montoya CA, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC, and Roy NC (2022) The effects of ruminant milk treatments on hippocampal, striatal, and prefrontal cortex gene expression in pigs as a model for the human infant. Frontiers in Neuroscience 16:937845. doi: 10.3389/fnins.2022.937845

**Chapter 6 Integration of changes in brain and blood variables in response to milk treatments in early postnatal life**

This chapter was written to support the findings of previous chapters and is not intended for publication.

**Chapter 7 Thesis discussion**

This chapter was written as a general discussion of the thesis and is not intended for publication.

# Chapter 1

# Thesis Introduction

The early years of childhood form the basis for physical, metabolic, emotional, cognitive, and social development and have a lasting impact on adult life. Although development starts *in utero*, the developmental events postnatally, from birth up to two-three years of life, are equally crucial.

The early postnatal period is marked by rapid brain development involving the increase of brain volume and cortical complexity, synaptogenesis, myelination, refinement of neural connections, and establishment of cognitive abilities (Knickmeyer et al., 2008; Tau and Peterson, 2009; Gilmore et al., 2012, 2018). In parallel with these substantial changes in the brain, the gut also undergoes profound growth, morphological changes, and functional maturation, including gut microbiota development (Jakoi et al., 1985; Koenig et al., 2011; Nagpal et al., 2017).

Studies in infants and rodent models have suggested that brain development during the critical period of early postnatal life is dependent on gut development (Sudo et al., 2004; Gareau et al., 2011; Heijtz et al., 2011; Clarke et al., 2013; Gao et al., 2019; Callaghan, 2020). For instance, one-year-old infants with a high relative abundance of the *Bacteroides* genus in their faeces had better cognitive performance in terms of receptive language and expressive language (Carlson et al., 2018). In contrast, infants with a low relative abundance of the *Faecalibacterium* genus showed lower cognitive performance (Carlson et al., 2018).

The developmental dependency of the brain on the gut in the early postnatal period can be attributed to the gut-brain axis (GBA), complex, bidirectional signalling, incorporating neural (e.g., vagus nerve (VN)), endocrine (e.g., glucagon-like peptide (GLP)), immune (e.g., microglia) and metabolic mediators (e.g., tryptophan (Trp) metabolites). For instance, a study had shown that *Lactobacillus rhamnosus* oral administration improved

emotional behaviour in mice with the VN intact, which was not the case when the mice were vagotomised (Bravo et al., 2011), suggesting the importance of vagal mediator in gut-brain communication.

Nutrition is increasingly recognised as one of the essential drivers of early postnatal brain development via modulating the GBA. A recent study on piglets has shown that human milk-associated gut microbial profiles were correlated with ileal, colonic and brain variables involved in barrier function, Trp metabolism and endocrine function (Charton et al., 2022). These correlations differed from those identified in groups fed infant formula (Charton et al., 2022). Another study in piglets showed human milk oligosaccharides (HMO)-associated gut bacterial profile were both positively or negatively correlated with short-term memory, which was mediated by hippocampal gamma-aminobutyric acid (GABA) neurotransmission-related genes and cortical volume (Fleming et al., 2021).

Although human breast milk is the best source of nutrition for infants, milk from other species, bovine, caprine and ovine milk, is used by formula manufacturers and households when breast milk is limited or unavailable. In this context, bovine milk is most used, while the use of milk from caprine and ovine species is steadily gaining momentum. These milk differ in their nutrient composition. Ovine milk has higher protein, lipid, and energy content than bovine and caprine milk (Park et al., 2007; Barlowska et al., 2011; Claeys et al., 2014). In contrast, caprine milk has higher oligosaccharide content and a more similar oligosaccharide profile to human milk compared to ovine and bovine milk (Van Leeuwen et al., 2020; Shi et al., 2021).

An *in vivo* study has highlighted the differences in gastric digestion between ruminants' milk; the gastric emptying rate of ovine and caprine milk proteins and lipids was faster than that of the bovine milk counterparts in piglets (Roy et al., 2022a). *In vitro* studies

have shown that fermentation of pre-digested ovine, bovine, and caprine milk using an infant faecal inoculum showed that the microbial composition of fermentation samples differs between milk types (Ahlborn et al., 2020; Gallier et al., 2020). The differences in nutrient composition, gastric dynamics and colonic microbiota profile between milk treatments observed in these studies might result in differences in the absorption, and metabolism of nutrients between milk treatments, thereby potentially influencing brain development. For instance, higher Trp content in ovine milk (Claeys et al., 2014; Rafiq et al., 2016) might result in higher levels of the neurotransmitter serotonin (5-HT) produced by enterochromaffin cells of the gut epithelium by utilising Trp as a precursor (Biggio et al., 1974; Gershon and Tack, 2007), which in turn might influence the brain neurotransmission via GBA (Clarke et al., 2013; Cowen and Sherwood, 2013). However, no studies have been conducted to understand the comparative effects of different ruminants' milk on early postnatal brain development via GBA.

Therefore, this thesis aims to understand better the effects of ruminants' milk used to make infant formula on early postnatal brain development via the gut-derived metabolites found in peripheral blood circulation.

The introduction presented in Chapter 1 sets the context of the thesis. Chapter 2 provides a comprehensive literature review covering the early life development of the brain and gut. Aspects include current knowledge of the brain and gut development during the prenatal years of life, and recent findings on the developmental interdependency between the gut and brain and their communication. The review also highlights the role of early life nutrition during the critical window of brain and gut development and the knowledge gaps in this field warranting further research.

# Chapter 2

# **Literature Review**

Part of the contents presented in this chapter has been published as a peer-reviewed paper:
**Jena A**, Montoya CA, Mullaney JA, Dilger RN, Young W, McNabb WC and Roy NC (2020) Gut-Brain Axis in the Early Postnatal Years of Life: A Developmental Perspective. Front. Integr. Neurosci. 14:44. doi: 10.3389/fnint.2020.00044

## 2.1 Early life brain development

The development of the brain is an organised, predetermined, and highly dynamic multistep process. It begins *in utero* following fertilisation and continues postnatally into human adolescence (Gibb and Kovalchuk, 2018). Brain architecture is shaped during the early postnatal period, and the foundation is set for perceptual, cognitive, and emotional abilities (Paterson et al., 2006). It is increasingly recognised as crucial for establishing cognitive and behavioural abilities that last a lifetime (Nelson et al., 2007). Recently, emphasis has been given to the first 1,000 days as an opportunity to influence cognitive outcomes in the child (Cusick and Georgieff, 2016). Studies elucidating brain development over this period are vital for research, clinical, educational, and social outcomes. For instance, data on brain development may be relevant for the early diagnosis of behavioural disorders like autism (Keehn et al., 2013; Wolff et al., 2015).

The critical brain developmental events include neurulation, neurogenesis, gliogenesis, neural migration, synaptogenesis, myelination, apoptosis, and synapse pruning (see reviews by (Andersen, 2003; Tau and Peterson, 2009; Davis, 2018)). In the prenatal period, the development of the brain is mostly influenced by genetic determinants, but in the early postnatal period, environmental factors take precedence. Hence, brain developmental events in the early postnatal period are particularly important, as less favourable environmental conditions can compromise the foundation of brain development and adversely impact later stages of life (McCrory et al., 2010).

### 2.1.1 General features

The human brain is considered the most advanced and complex of all biological systems, containing around 100 million neurons and 10 times more glial cells. Neurons are specialised cells responsible for receiving and transmitting signals, while glial cells

provide support and protect (e.g., supply nutrients) neurons. Neurons are similar to other cells in the body but with a few specialised features. Neurons have a cell body called the soma, which has a unique structure for receiving and sending signals called dendrites and axons, respectively. Dendrites are specialised in receiving signals from other neurons, whereas the axon's role is to transfer the signals outward from the cell body to the axon terminal. Axons are wrapped in a fatty substance called myelin, making transmitting of signals between neurons more efficient. The neuronal body forms synapses, a chemical junction between the axon of one neuron and the dendrite of another neuron. Once the synapse is formed, signals are transferred between two neurons by chemical messengers called neurotransmitters. Glial cells, on the other hand, are shorter than neurons and lack dendrites and axons.

The structure of the human brain is walnut-shaped and is divided into regions that perform different functions (Figure 2-1). The brain has a deeply folded cerebral cortex representing around 80% of the brain's mass. The mature brain has a characteristic pattern of folds (the sulci) and ridges (gyri) that allows the brain to fit into the cranial vault. Also, a ubiquitous characteristic of the vertebrate brain is segregation into the grey and white matter. Grey matter contains local networks of neurons that are connected by dendrites and mostly non-myelinated local axons. White matter contains long-range axons that perform communication via often myelinated axons.

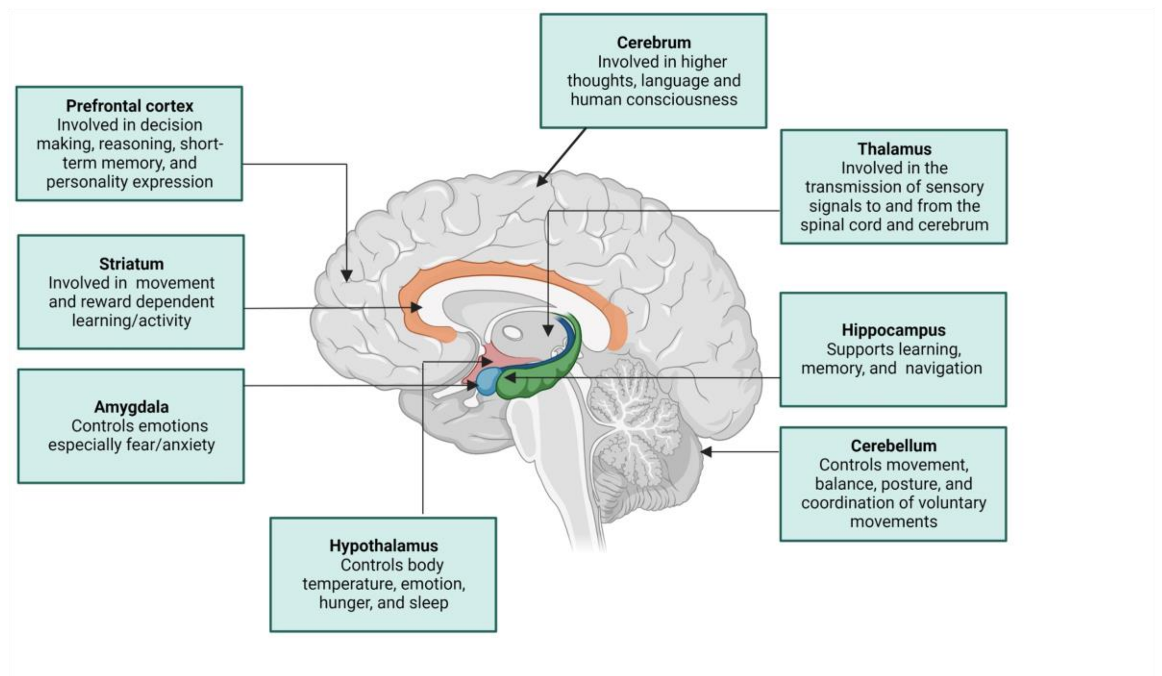



**Figure 2-1 Major regions of the human brain and their functions.**

Figure created with Biorender.com.

## 2.1.2 Prenatal development

The prenatal period includes the embryonic and foetal periods. The embryonic period of brain development begins at conception and extends until week 8 of gestation (Stiles and Jernigan, 2010). Brain development events during the embryonic period include the differentiation of neural progenitor cells and the formation of the neural tube (Tau and Peterson, 2009). The embryonic period is followed by the foetal period of human development, which starts at week 9 of gestation and extends until the end of gestation (Stiles and Jernigan, 2010).

The developmental events of the brain during the foetal period involve the proliferation and elimination of neural elements (Stiles and Jernigan, 2010). During the foetal period, brain development undergoes a series of complex, sequential, and yet overlapping events like the production of neurons from the innermost part of the neural tube (neuron

proliferation), migration of neurons from their site of origin to different locations within the central nervous system (CNS) (neuron migration), increase of neuron size and formation of neuronal structures axon and dendrites (neuron differentiation), formation of connections between the axon of one neuron and the dendrite of another neuron (synaptogenesis), wrapping of axons of neurons with myelin sheath (myelination), and removal of overproduced and immature neurons (apoptosis) and synapses (synaptic pruning) (Tau and Peterson, 2009). Synaptogenesis, myelination, and regressive events (apoptosis and synapse pruning) start in the prenatal period and continue throughout the postnatal period (Tau and Peterson, 2009; Tierney and Nelson, 2009).

### 2.1.3 Postnatal development

In the postnatal period, neurogenesis (formation of neurons) continues to a limited degree in the olfactory bulb (Bergmann et al., 2012) and hippocampal dentate gyrus throughout life (Boldrini et al., 2018). Unlike neurogenesis, gliogenesis (formation of glia) peaks during the first year of life and continues until adolescence (Semple et al., 2013; Reemst et al., 2016; Allswede and Cannon, 2018).

Glial cells have three significant cell subtypes within the brain: microglia, astrocytes, and oligodendrocytes, each with different developmental timelines. The microglia regulate neurogenesis and synaptic refinement; astrocytes support the formation and plasticity of the synapse, while the oligodendrocytes form myelin (Eroglu and Barres, 2010). The proliferation of microglia peaks in the first two weeks after birth and continues until the first month after birth (Budday et al., 2015). The proliferation of astrocytes and oligodendrocytes peaks before birth and continues until fifteen months of age and adulthood, respectively (Allswede and Cannon, 2018; Davis, 2018). Apoptosis of neuronal cells is largely completed *in utero*. However, apoptosis of the glial cell population continues to occur in the first few months after birth (Tau and Peterson, 2009;

Stiles and Jernigan, 2010). Oligodendrocytes undergo apoptosis to control myelin production during the initial stage of myelination (Caprariello et al., 2015).

Synaptogenesis peaks across most of the regions of the brain in the early years of postnatal life (Huttenlocher and Dabholkar, 1997). Synaptogenesis peaks at different times in different regions of the brain, such as in the areas of the cerebral cortex, where heterogeneity in synaptogenesis is well documented (Huttenlocher and Dabholkar, 1997). The infant's brain has almost double the number of synapses compared to the adult brain, and their abundance is reduced by synaptic pruning, which is pronounced during childhood to adolescence (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997). Together the formation and retraction of synapses shape the neural connections in the brain.

The cerebral cortex is divided into three functionally distinct areas, namely, sensory areas (e.g., visual cortex, auditory cortex), motor areas (e.g., motor cortex), and association areas (e.g., prefrontal cortex). Synaptogenesis in the visual cortex (present in the occipital lobe) peaks at around six months of age (Huttenlocher, 1999), whereas in the auditory cortex (temporal lobe), it peaks around three months of age, and in the prefrontal cortex (present in the frontal lobe) around three years of age (Huttenlocher and Dabholkar, 1997). Hence, this developmental pattern indicates synaptogenesis peaks first in the sensory areas and later in the association areas, from a posterior to an anterior direction (Huttenlocher and Dabholkar, 1997; Giedd et al., 1999). Furthermore, the communication across synapses is facilitated by neurotransmitters whose abundance increases concomitantly with synaptogenesis (Herlenius and Lagercrantz, 2004).

Myelination is a critical cellular event for the development of the brain, particularly for enhanced neuronal activity and communication. Myelination peaks during the first three

years of life and continues until the second and third decades years of life (Giedd et al., 1999). Like synaptogenesis, myelination occurs first in the sensory areas, followed by association areas from a posterior to an anterior direction (Volpe, 2000; Barkovich, 2005). Hence, the developmental pattern of synaptogenesis and myelination indicates areas with critical functions in early postnatal life, thus necessitating an earlier requirement for maturation (Huttenlocher and Dabholkar, 1997; Barkovich, 2005).

The brain undergoes significant structural development in the first two years of life (Casey et al., 2000). At birth, the total brain volume is 36% of an adult brain, reaching around 70% by the first year of age and 80% by the second year (Knickmeyer et al., 2008). The cortical volume also increases by 88% in the first year and 15% in the second year (Knickmeyer et al., 2008). The cortical thickness and surface area determine cortical volume, and these determinants also change in the first two years of life. The increase in cortical thickness and surface area is 31% and 76.4% in the first year of life and 4.3% and 22.5% in the second year (Lyall et al., 2015). Regional differences in cortical thickness and surface areas are also observed (Shaw et al., 2008; Lyall et al., 2015; Remer et al., 2017). The volume of the thalamus and amygdala increases by 130% and 14% in the first and second year, respectively (Knickmeyer et al., 2008). The hippocampus grows slowly in the first year but increases rapidly in the second year, likely linked to the increasing complexity of spatial working memory and path integration when children become more mobile (Wolbers et al., 2007; Gilmore et al., 2012).

Concurrent with rapid cellular and structural brain growth is an equally rapid development of brain functions in the first years of postnatal life. The brain's functional networks are present *in utero* but continue to develop in the early postnatal period (Gao et al., 2015). Primary sensory-motor and auditory networks are the first to develop, followed by visual, attention, and default mode networks, and finally, the executive

control networks begin to emerge (Gao et al., 2015). Different functional networks are activated during different cognitive tasks performed by infants, such as distinguishing different voices, recognising faces, object permanence, etc. (Paterson et al., 2006).

Changes in the structural and functional networks of the brain contribute to the development of cognitive abilities (e.g., perception and memory) in the first years after the birth of infants. These developmental events are mainly affected by external factors (diet, early life experiences) (Nelson et al., 2007; Deoni et al., 2018). Therefore, any positive and negative alterations of these external factors can either enhance or compromise the development of the brain.

Within the body, the early life development of the brain is co-dependent on the development and appropriate functioning of many organs. For example, it is recognised that the gut plays one of the most significant roles in shaping brain development.

## 2.2 Early life gut development

The gut is one of the most crucial organs during the early life development of humans. Postnatally, the survival of an individual is dependent on gut development. It digests and absorbs nutrients that are then delivered to the body's organs for maintenance, growth, and development. *In utero*, the foetus gets nutrients from the maternal blood via the placenta (Salafia et al., 2007), but after birth, the infant begins enteral nutrition with the uptake of breast milk (Sangild et al., 2000). This shift from parenteral to enteral nutrition requires a developed gut before birth (Sangild et al., 2000). At birth, the tube is fully formed with the required motility functionality to ensure the survival of the infant on the mother's breast milk, independent of placental nutrition (Grand et al., 1976). However, the gut continues to mature in structure and function postnatally.

### 2.2.1 General features

The gut carries out a series of functions: the passage/transit of food through the lumen, digestion of food components; absorption of nutrients; and control of all these functions by local, nervous, and hormonal systems (Hall, 2016). The gut consists of the mouth, oesophagus, stomach, and small and large intestines, performing different functions (Figure 2-2). In the mouth, food mixes with saliva with the action of the tongue to form a bolus. With swallowing, the bolus passes down to the oesophagus into the stomach. The bolus is mixed with the gastric secretions to form chyme, which then enters the small intestine, where most digestion and absorption occur. In the duodenum, the first segment of the small intestine, chyme is mixed with pancreatic enzymes and bile from the liver for further digestion. The jejunum, the second segment of the small intestine, largely contributes to the absorption of nutrients into the bloodstream. Ileum, the final segment, contributes to the digestion of vitamins and bile salts. The resulting undigested food passes to the large intestine, which comprises the caecum, colon, and rectum, where absorption of water and osmoregulatory agents (e.g., Na, K) and digestion of the food remnants and stool formation occur.

Unlike other peripheral organs, the gut has a dedicated nervous system called the enteric nervous system (ENS). The ENS is embedded along the gut wall and consists of a network of neurons that mainly resides within two major ganglionated plexuses (Furness, 2012). The myenteric plexus lies in the muscular propria layer, and the submucosal plexus is in the submucosa layer. The regulation and coordination of muscular and secretory activities by the ENS are required for digestion and absorption (Rao and Gershon, 2016). Along with innervation by the ENS, the gut also receives nerve supply from the CNS that can enhance or inhibit gut functions. Hence, this relationship between the ENS and CNS is crucial for optimised gut functions.

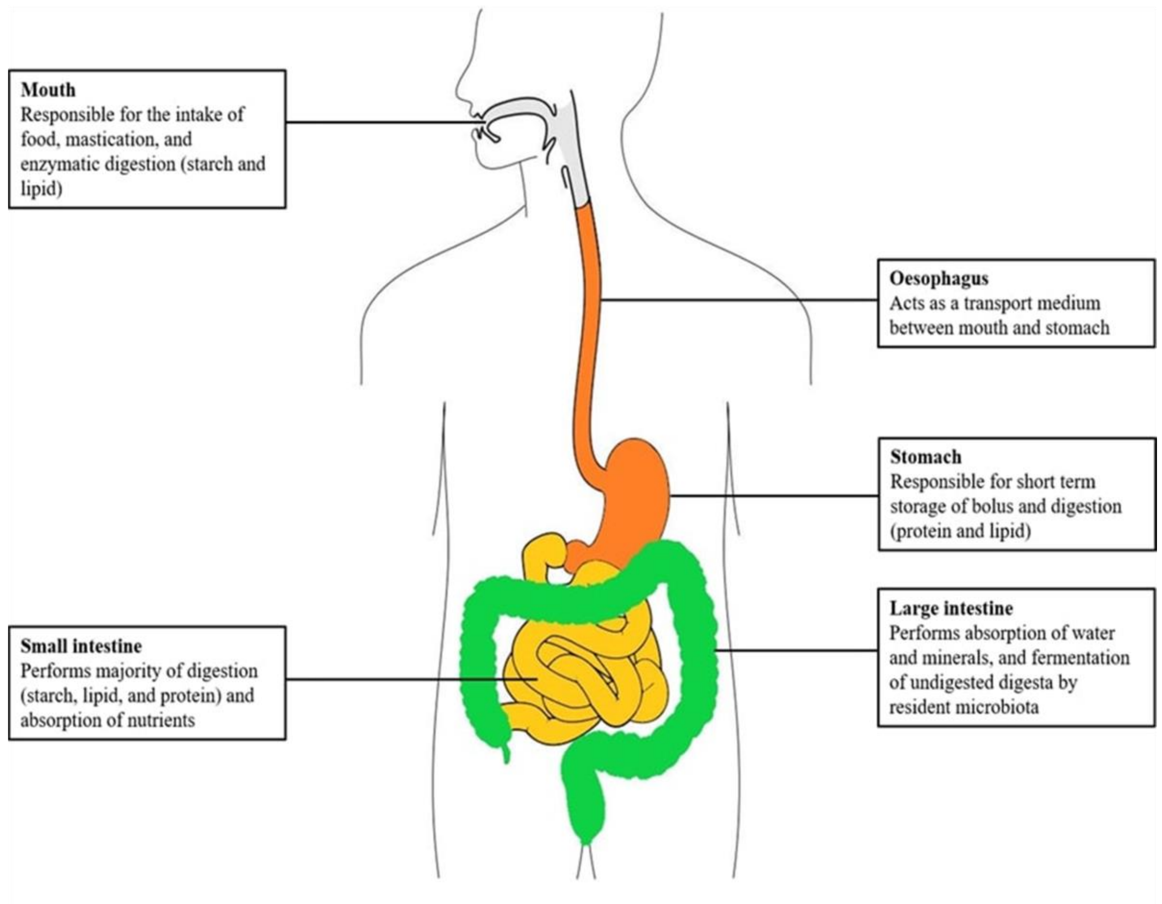



**Figure 2-2 Overview of the human gut.**

Figure created with Biorender.com

## 2.2.2 Prenatal development

In the prenatal period, gut development begins with tube formation and completes with a developed motility function (Grand et al., 1976; Montgomery et al., 1999; Neu, 2007). Between weeks 3-4, the formation of the foregut, midgut, and hindgut from the embryonic germ layer occurs. Between weeks 6-12, the gut tube dramatically increases in length and diameter. Formation of villi and crypts between weeks 9-11 marks the end of the first stage of gut development. Enzymes (ATPase, alkaline phosphatase, sucrase, maltase, and lysosomal enzymes) and hormones (peptide YY (PYY) and gastrin) are detected in the

foetal tissues between weeks 8-24. Amniotic fluid swallowing by the foetus takes place by weeks 16-17. Finally, maturation of the motility function of the gut occurs between weeks 27-33.

### 2.2.3 Postnatal development

The structural and functional maturation of the gut postnatally occurs in response to early-life food (breast milk and/or formula) (Zhang et al., 1998; Jensen et al., 2001). Structural maturation includes changes in size and anatomical features. Postnatally, the oesophagus, stomach, and small and large intestines continue to grow (Weaver et al., 1991; Xu, 1996). The postnatal period is also marked by a decline in epithelial permeability (Jakoi et al., 1985; Jakobsson et al., 1986; Drozdowski et al., 2010). After birth, the small intestine is permeable to macromolecules (e.g., immunoglobulin G) in breast milk (Jakoi et al., 1985). Within the first few days, the small intestine's permeability to macromolecules is reduced, which results in the cessation of macromolecule transport paracellularly (Jakoi et al., 1985). The exact timing of permeability reduction in humans remains unknown. Still, studies in piglets and rats suggest that barrier closure happens in the first two days after birth (Weström et al., 1984) and postnatal week three (Sureda et al., 2016), respectively. Villi development is primarily completed at birth, whereas a rapid increase in crypt depth and crypt cell proliferation in the small intestine also occurs in the first years of life, increasing the surface area for nutrient absorption (Thompson et al., 1998; Cummins and Thompson, 2002). Maturation of ENS in terms of neuronal morphology (e.g., dendritic and axonal structure), types of neurons (e.g., cholinergic and nitrergic), neurally-mediated motility patterns in different regions of the gut also occurs during the postnatal period, as reviewed by (Foong, 2016).

*In utero,* the gut of the foetus is exposed to amniotic fluid, which contains 98% water and 2% protein, sodium, chloride, and $CO_2$ (i.e., low nutrient content) (Bonsnes, 1966).

Immediately after birth, the infant is introduced to colostrum, which is rich in proteins (e.g., lactoferrin and lactoperoxidase), immunoglobulins, and growth factors (e.g., epidermal growth factor, vascular endothelial growth factor) (Ballard and Morrow, 2013; Godhia and Patel, 2013). The infant's gut undergoes further functional development to adapt to complex and more diverse nutrient profiles postnatally (Hampson, 1986; Thompson et al., 1998). The activity of the enzymes enterokinase (protein hydrolysis), gastric lipase (lipid hydrolysis), and lactase (carbohydrate hydrolysis) increases gradually after birth (Antonowicz and Lebenthal, 1977; Moreau et al., 1988; Shulman et al., 1998) to facilitate the digestion of complex food structures. Functional maturation of the gut in the postnatal period also includes the establishment of the gut microbiota.

#### 2.2.3.1 Microbial colonisation

The colonisation of microbes in the gut begins at birth, and significant changes in the composition of gut microbiota occur until about three years (Koenig et al., 2011; Yatsunenko et al., 2012). However, the literature suggests the presence of microbes *in utero.* This view arises from the fact that microbes have been detected in the meconium (i.e., the first stool of the infant after birth), amniotic fluid, and placenta (Aagaard et al., 2014; Ardissone et al., 2014; Urushiyama et al., 2017; Shi et al., 2018). Ardissone et al., 2014 showed that approximately 61% of the microbial population in meconium was similar to that of the amniotic fluid, suggesting that microbes in the meconium originate from swallowing amniotic fluid by the foetus (Ardissone et al., 2014). The viability of microbes *in utero* remains debated in the scientific community, and the problem of contamination artefacts is discussed among researchers. However, recent mouse studies showed viable bacteria in the foetal gut, uterus, and placenta, suggesting the possibility of viable bacteria in a human foetus (Younge et al., 2019). Therefore, more studies on *in utero* colonisation are warranted to challenge the accepted sterile womb paradigm.

In the postnatal period, the microbial colonisation of the infant's gut follows a succession of steps. Studies of the gut microbiota in the infant are limited to faecal samples. Stool samples are a proxy for the microbial population of the large intestine but may not represent it accurately. During the first few weeks after birth, the gut microbiota of infants is dominated by facultative anaerobes like members of the *Enterobacteriaceae* family (Palmer et al., 2007; Matsuki et al., 2016; Nagpal et al., 2017), which are likely originating from the mother's vagina and skin (Palmer et al., 2007; Lozupone et al., 2013). At around six months, strict anaerobes, including bacteria of the *Bifidobacterium, Clostridium*, *and Bacteroides* genera, dominate the composition (Nagpal et al., 2017). At around three years of age, the microbiota profile shows a high degree of resemblance to that of adults (Palmer et al., 2007; Koenig et al., 2011; Yatsunenko et al., 2012) and is represented almost entirely by strict anaerobes like the *Clostridium* cluster XIVa and IV and *Prevotella* genus (Nagpal et al., 2017).

The gut microbial community consists not only of bacteria but also phage, archaea, and fungi. However, most studies have focused on bacterial colonisation of the gut in infants, and much less is known about other kingdoms of life. According to the available knowledge, bacteriophage, mainly of the Caudovirales order and *Microviridae* family, the archaea *Methanobrevibacter smithii,* and the fungi *Candida albicans* are the most predominant non-bacterial organism in the infant's gut during the first years of life (Palmer et al., 2007; Smith et al., 2013; Heisel et al., 2015; Lim et al., 2015, 2016; Schei et al., 2017; Ward et al., 2017).

## 2.3 Parallel development between the gut and brain

The majority of the development of the gut and brain occur in parallel, but their development is asynchronous in terms of attaining peak and maturity. For instance,

microbial colonisation, tissue structural maturation, and ENS maturation coincide with the refinement and remodelling of brain neural circuits and cognitive development in the first years of life (Figure 2-3). There is increasing evidence that the colonisation of the gut by the microbiota appears to have a parallel developmental trajectory to the brain for up to three years. For example, Carlson et al., 2018 showed that infants with a high relative abundance of *Bacteroides* spp. in their stools had better cognitive performance in terms of receptive language and expressive language. In contrast, infants with a high level of *Faecalibacterium* spp. in their stools had lower cognitive performance (Carlson et al., 2018). Another study in infants showed a positive association between the alpha diversity of the faecal microbiota and the functional connectivity between the supplementary motor area and the inferior parietal lobule (areas associated with cognitive outcomes) of the brain (Gao et al., 2019).

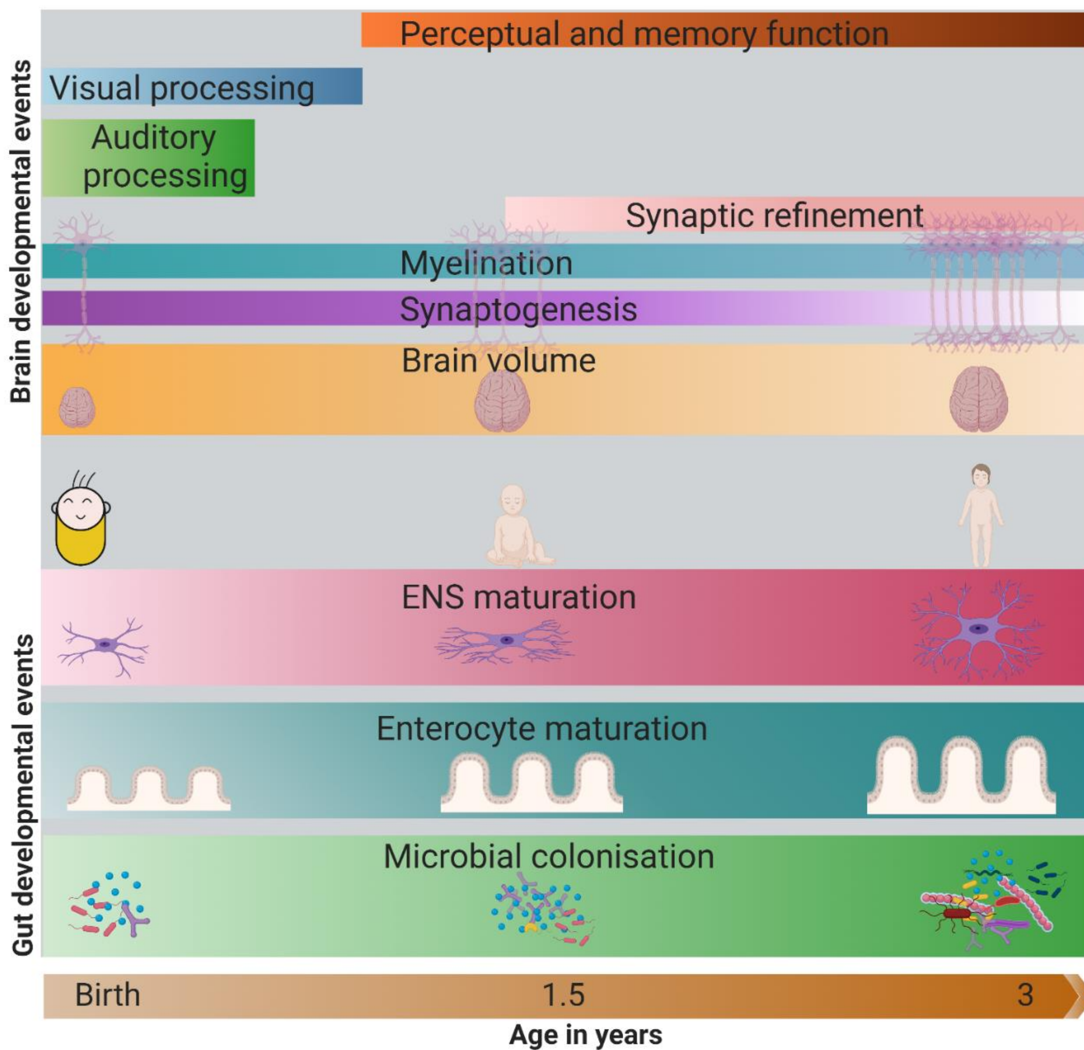



**Figure 2-3 Parallel development of the gut and the brain in the first three years of life.**

In the gut, increases in microbial abundance and diversity, enterocyte maturation (change in crypt and villi structure), and ENS maturation (change in nerve density, type of neurons) occur rapidly in the first three years of life. Meanwhile, the brain also develops rapidly, with the change in volume (peak in the first year), synaptogenesis, myelination, synaptic refinement, and establishment of cognitive abilities like auditory and visual processing, perception, and memory. The darkness of the colour represents the intensity/peak of the developmental event. ENS, enteric nervous system. Figure created using Biorender.com.
Note: The developmental timing of the cellular events may vary across different brain regions.

Evidence from rodent studies has also provided insights into the correlation between changes in gut microbiota and brain function in early postnatal life. Germ-free (GF) mice displayed altered anxiety responses, abnormal motor activities, enhanced stress responses, and memory dysfunction (Sudo et al., 2004; Gareau et al., 2011; Heijtz et al., 2011). Interestingly, when GF mice are conventionalised with faecal microbiota obtained from specific pathogen-free (SPF) mice in adulthood rather than early postnatal life, anxiety-like behaviour associated with altered synaptic-related proteins and neurotransmitter turnover persist (Sudo et al., 2004; Heijtz et al., 2011). These findings suggest that specific changes in brain structure and function cannot be reversed beyond a critical window in the early postnatal period (Sudo et al., 2004; Heijtz et al., 2011).

Additionally, adult GF mice exhibit a decreased production of the neurotransmitter 5-HT in the gut compared to conventionally raised and SPF adult mice (Reigstad et al., 2015; Yano et al., 2015). 5-HT is also produced in the brain (c.f., Section 2.4.4.1). It is well known that brain-derived 5-HT is associated with mood regulation, learning, and memory (Cowen and Sherwood, 2013; Carhart-Harris and Nutt, 2017), but whether changes in gut-derived 5-HT regulate these brain functions remains to be confirmed.

A study by Collins et al., 2014 showed that at three days of age, the development of the myenteric plexus of the ENS was structurally abnormal in GF mice compared to that of SPF mice. The myenteric plexus showed decreased nerve density and ganglionic size but increased nitrergic neurons in the GF mice (Collins et al., 2014). Whether these functional changes in the gut translate into cognitive outcomes, remains unknown, but it is plausible that there is an interdependency between the establishment of the gut microbiota, the ENS and the development of the brain.

It is important to note that findings from studies in rodent models may not be reproducible in humans, as there is a marked difference between rodents and humans in terms of the developmental patterns of the gut and brain. Rodents are born with a relatively underdeveloped gut, and most functional development occurs in the postnatal period (Searle, 1995; Drozdowski et al., 2010; Guilloteau et al., 2010). The timing of brain developmental events is also different between humans and rodents (Pressler and Auvin, 2013). However, the anatomy and physiology of the gut, brain growth, and developmental patterns of both organs in piglets share a greater similarity to humans than in other non-primate models like rodents (Guilloteau et al., 2010; Mudd and Dilger, 2017).

Most studies of gut and brain development have mainly focused on the role of the gut microbiota. The gut undergoes developmental changes not only in terms of microbiota but also enzyme activity, gastric secretions, intestinal permeability, and increased surface area for absorption of nutrients (i.e., crypt-villi structural modification) (c.f., Section 2.2.3). How these changes in the gut mucosa affect brain outcomes remains mostly unknown. For instance, an increase in the surface area of absorption of nutrients over this period could result in increased availability of nutrients for absorption and less for the microbiota. The result could be a profile of different neuroactive metabolites in the gut and potentially contributing to specific cognitive outcomes.

## 2.4 Mechanism of communication between the gut and brain

The gut and the brain are connected through a complex network of signalling pathways collectively termed the GBA (Carabotti et al., 2015). The communication between the gut and brain is bidirectional and mediated by neural, endocrine, immune, and metabolic mediators. In the last decade, the role of gut microbiota in the GBA has been extensively

assessed. Consequently, the term has been extended to microbiota-GBA. Here, the term GBA includes microbiota.

The GBA has been studied using “top-down” and “bottom-up” approaches. However, the modulation of the gut functions by the brain (top-down approach) is well established by preclinical and clinical evidence. For instance, modulation of motility, secretion (HCl acid in the stomach, bicarbonates in pancreatic juice, and mucus by goblet cells), and mucosal immune responses in the gut are controlled by the brain as reviewed elsewhere (Rhee et al., 2009).

The modulation of brain functions by gut-derived molecules (bottom-up approach) involves different signalling pathways (Figure 2-4). The importance of the GBA is increasingly recognised in physiological (e.g., gut homeostasis) and pathological conditions (e.g., mood disorders, obesity, autism) and have been extensively reviewed elsewhere (Mayer, 2011; Agustí et al., 2018; Liu and Zhu, 2018; Martin et al., 2018). However, understanding GBA during the parallel development of the gut and the brain in the early postnatal period is limited.

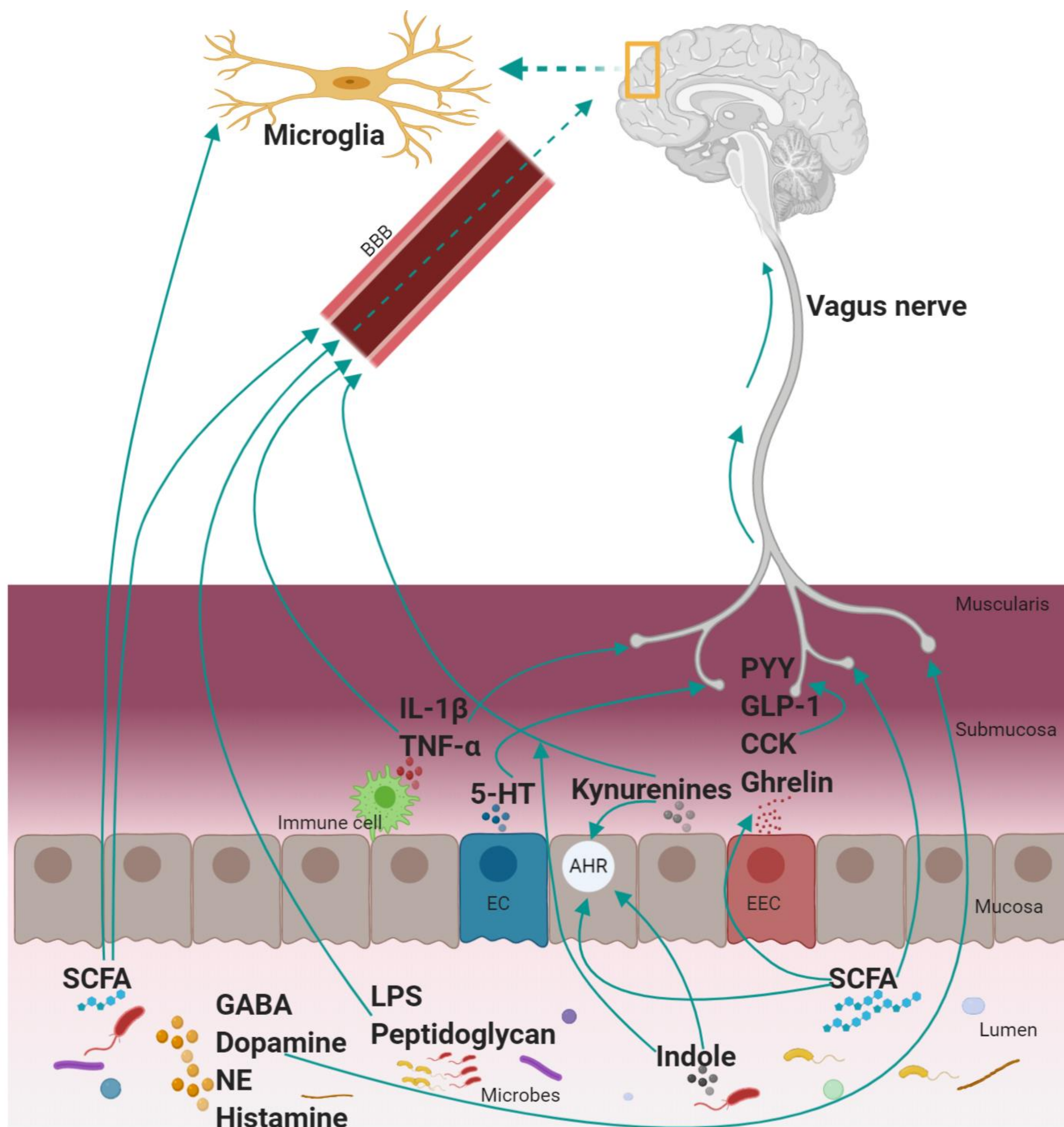



**Figure 2-4 Mechanism of communication between the gut and the brain.**

A myriad of mediators is involved in the complex communication between the gut and the brain. These include neural (vagus nerve), endocrine (hormones; PYY, GLP-1, CCK, ghrelin), immune (cytokines (IL-1β, TNF-α), microglia, microbial antigenic component (LPS, peptidoglycan), and metabolic (Trp metabolites (kynurenines, 5-HT, indole), SCFA, neurotransmitters (GABA, dopamine, NE, histamine)) mediators. The mode of action of these mediators is by activating the vagus nerve or crossing the BBB to communicate with the brain directly. SCFA regulate other mediators (EEC to produce hormones, microglia maturation, AHR activation; an essential receptor for Trp metabolites (produced by the host and microbiota). GLP-1, glucagon-like peptide-1; PYY, peptide YY; CCK, cholecystokinin; TNF-α, tumour necrosis factor-α; IL-β, interleukin-β; GABA, gamma-aminobutyric acid; NE, norepinephrine; SCFA, short-chain fatty acids; EEC, enteroendocrine cells; Trp, tryptophan; LPS, Lipopolysaccharides; BBB, blood-brain barrier; AHR, aryl hydrocarbon receptor; EC, enterochromaffin cell; 5-HT, serotonin. Figure created with Biorender.com.
Note: Kynurenines include kynurenine and downstream metabolites of the kynurenine pathway and not all the kynurenines can cross the blood-brain barrier.

### 2.4.1 Neural mediators

The VN is the longest nerve in vertebrates and innervates many visceral organs like the heart, lungs, and gut (Bonaz et al., 2018). It has a vital role in many functions, such as digestion, immune responses, heart rate, and controlling mood (Breit et al., 2018). The VN also plays a crucial role in facilitating neural signals between the gut and the brain (Bravo et al., 2011). It is the principal component of the parasympathetic nervous system and is composed of 80% afferent and 20% efferent fibres (Bonaz et al., 2018). The afferent fibre carries information from the gut to the brain, and the efferent nerve fibre carries information from the brain to the gut. The efferent fibre regulates motility and glandular secretion in the gut, possibly by interacting with the ENS, mainly by cholinergic activation via nicotinic receptors (Garza et al., 2009; De Jonge, 2013). Over the last decade, the vagal afferent pathways have been increasingly recognised as sensors of hormones, cytokines, and metabolites produced in the gut with potential consequences for brain function and behaviour. The afferent pathway is also involved in activating and regulating the hypothalamic-pituitary-adrenal axis, a principal component of the physiological stress system and a key mediator of the GBA during stress, as reviewed elsewhere (De Weerth, 2017).

Vagal afferent fibres are located in all layers of the gut but do not cross the mucosal layer outwardly (Wang and Powley, 2007). Thus, they cannot sense the luminal contents directly but indirectly through the diffusion of microbial metabolites such as short-chain fatty acids (SCFA) (Lal et al., 2001) or via enteroendocrine cells (EEC) (Li et al., 2000). The EEC represent about 1% of epithelial cells in the gut and forms the body's largest endocrine organ (Mayer, 2011). These cells can sense luminal contents, and in response, they produce and release molecules (e.g., a variety of hormones and 5-HT) that bind to receptors expressed on afferent endings (Egerod et al., 2012, 2018). A study showed that

the administration of *Lactobacillus rhamnosus* improved depression and anxiety-like behaviour in mice with the VN intact (Bravo et al., 2011). However, these effects were not observed in vagotomised mice, suggesting the importance of the VN in modulating brain functions in response to a specific bacterium (Bravo et al., 2011).

In early postnatal life, the VN is not fully functional. During birth, VN was only partially myelinated (Porges and Furman, 2011). As discussed before (c.f., Section 2.1.3), nerve myelination continues in the postnatal period, which also holds for the VN. Development from partially to fully myelinated VN starts at approximately 24 weeks of gestation and continues until adolescence (Sachis et al., 1982; Porges and Furman, 2011). However, a faster VN myelination rate was observed from 32 weeks of gestation until six months after birth (Sachis et al., 1982). This observation suggests accelerated transmission of signals between the gut and brain during this period, likely due to the consumption of breast milk or formula by infants. Milk is an essential source of long-chain polyunsaturated fatty acids (LCPUFA) (e.g., docosahexaenoic acid and arachidonic acid), sphingolipids (e.g., sphingomyelin (SM)), phospholipids (e.g., phosphatidylcholine (PC)), and cholesterol, which are all essential for myelin sheath synthesis and development (Deoni et al., 2018). However, the effects of breast milk or substitutes on the myelination of the VN are poorly understood.

### 2.4.2 Endocrine mediators

The hormones produced by EEC are essential mediators of the GBA. Ghrelin, GLP-1, cholecystokinin and PYY are produced and released by EEC in response to the food intake (Egerod et al., 2012; Latorre et al., 2016). These hormones regulate food intake, satiety, gastric emptying, and energy balance by transmitting signals between the gut and the brain, as reviewed elsewhere (Raybould, 2007; Cong et al., 2010; Holzer and Farzi, 2014). Ghrelin is mainly released by the stomach, which stimulates gastric emptying,

regulates appetite, and increases the release of growth hormone by the pituitary gland (Kojima et al., 1999; Sun et al., 2004). Cholecystokinin and GLP-1 are produced in the small intestine, inhibiting gastric emptying and reducing food intake (Liddle, 1997; Holst, 2007). The sites of production of PYY are the ileum and the colon, which decrease gastric motility, improve glucose homeostasis, and induce satiety (De Silva and Bloom, 2012).

Studies have shown that gut hormones are crucial role in regulating emotion and mood. For instance, ghrelin reduces anxiety-like and depressive-like symptoms of chronic stress (Lutter et al., 2008), whereas high PYY, mimicking its postprandial plasma concentration, promotes hedonic behaviour (Batterham et al., 2007). However, it remains to be proven that these effects occur in healthy conditions. Various gut hormones are produced in normal physiological conditions, and the effect of one hormone is possibly counterbalanced by others. For instance, GLP-1 enhances anxiety-like behaviour (Möller et al., 2002; Gulec et al., 2010), whereas GLP-2 could attenuate depression-like behaviour (Iwai et al., 2009). These hormones regulate the signalling between the gut and the brain, most likely by activating the receptors in the vagal afferent fibre (Egerod et al., 2018; Okada et al., 2018).

Nutrition in early postnatal life is known to influence the production of gut hormones. For example, infant-fed with formula during the first six months of age had higher ghrelin and lower PYY blood concentrations compared with infants fed breast milk over the same period (Breij et al., 2017). However, studies on the associations between changes in gut hormones and brain development in response to infant nutrition remain elusive.

### 2.4.3 Immune mediators

The constituents of the immune system, immune cells and signalling molecules act as important intermediaries in the GBA. Microglia, the tissue-resident immune cells in the

brain, have increasingly been recognised as a significant neuroimmune player of the GBA and in early life brain development (Erny et al., 2015). For instance, the microglia regulates neurogenesis and synaptic refinement by phagocytosing excess neurons and synapses (Schafer et al., 2012; Cunningham et al., 2013). Regulation of neurogenesis is crucial for ensuring that this process does not exceed neurons' demands of the developing brain and ultimately aids brain organisation (Cunningham et al., 2013). Synaptic refinement is essential for shaping the neural circuitry by eliminating the redundant synapses during postnatal brain development (Wu et al., 2015). A study by Erny et al., 2015 showed that the microglia in adult GF mice have abnormal morphology and density and altered cell proportions (e.g. dendrite length) compared to SPF mice. These adverse effects were partially rectified when adult GF mice were colonised with complex microbiota, suggesting a role for the microbiota in microglia maturation and function (Erny et al., 2015). It is important to note that the oral administration of a mixture of SCFA (acetate, propionate, and butyrate) (c.f., Section 2.4.4.2) was sufficient to drive the maturation of the microglia in GF mice (Erny et al., 2015). However, the mechanism underlying the maturation of microglia by SCFA remains to be determined. Evidence from these studies points to a relationship between the microbiota and the microglia that could be important in the immune-mediated aspects of the GBA and brain development in early postnatal life.

The signalling molecules of the immune system (e.g., cytokines) also participate in the GBA, possibly by two mechanisms: binding to VN receptors or transport across the blood-brain barrier (BBB). Evidence shows that the afferent VN fibre has receptors for the cytokine interleukin-1β (Ek et al., 1998). This cytokine can trigger its production and other pro-inflammatory cytokines that induce neuroinflammation (Shaftel et al., 2007). Tumour necrosis factor-α can cross the BBB (Gutierrez et al., 1993), resulting in

neuroinflammation and dysfunction in the brain (Seleme et al., 2017). Bacterial peptidoglycan (outermost covering of gram-positive bacteria) derived from resident commensals could also cross the BBB under physiological conditions, thereby influencing brain development and social behaviour in three-day-old mice (Arentsen et al., 2016)

Another study in rats has shown that lipopolysaccharides (LPS) from the surface of gram-negative bacteria can also cross the BBB (Vargas-Caraveo et al., 2017). Studies in mice have shown that intraperitoneal injection of LPS decreased novel object exploratory behaviour by impairing continuous attention and curiosity toward objects (Haba et al., 2012). LPS can bind to the toll-like receptor 4 expressed on the microglia (Laflamme and Rivest, 2001) and afferent VN (Hosoi et al., 2005). However, the relationship between LPS-driven immune activation and alteration of behaviour remains to be established.

The immune system in the early postnatal period undergoes the most rapid and radical changes compared with other systems in the body (Goenka and Kollmann, 2015). Commensal microbiota drives normal immune stimulation and maturation (Kamada et al., 2013; Olin et al., 2018). In infants, the cells of the innate immune system (e.g., monocytes, macrophages) are mostly developed prenatally, but their functions remain less developed in the newborn (Simon et al., 2015). This lower activity could be to avoid unnecessary immune reactions of continuous developmental remodelling (Prabhudas et al., 2011; Franchi et al., 2012). The cells of the adaptive immune system (e.g., B and T cells) are low in number and are functionally immature in infants (Tasker and Marshall-Clarke, 2003; Haines et al., 2009), which is most likely due to limited exposure to antigens required to develop an immune memory (Prabhudas et al., 2011). With the development of immune cells in early postnatal life, the level of their secretory products (i.e., cytokines) can also change over time (Corbett et al., 2010). This dynamic nature of the

immune mediators in early postnatal life likely contributes to brain development and associated behaviour.

## 2.4.4 Metabolic mediators

Metabolites are low molecular weight compounds, typically under 1,000 Da, which are reactants, intermediates, or products of enzyme-mediated biochemical reactions. Metabolites play essential roles in the GBA and can have either direct or indirect (e.g., interaction with a neural mediator) effects on brain function. The host, the gut microbiota, or their interactions can produce metabolites. Among various metabolites produced in the body, Trp metabolites, SCFA, and neurotransmitters are increasingly recognised as potential mediators of the GBA.

### 2.4.4.1 Tryptophan metabolites

Trp is an essential amino acid for synthesising body proteins and is a precursor to several metabolites. Once absorbed, Trp can be metabolised in enterocytes and hepatocytes, thereby reducing its availability to the rest of the body, including the brain (Waclawiková and El Aidy, 2018). Trp is metabolised through different pathways (hydroxylation and kynurenine) in the gut mucosa, producing neuroactive compounds (Bender, 1983) that are important for the GBA.

The hydroxylation pathway generates two important metabolites, 5-HT and melatonin, that participate in the GBA (Bender, 1983). The neurotransmitter 5-HT is involved in gut functions such as gastric secretion and motility (Gershon and Tack, 2007), and in the brain, it regulates mood and is involved in cognitive and behavioural functions (Cowen and Sherwood, 2013; Carhart-Harris and Nutt, 2017). About 95% of the total 5-HT in the body is synthesised by enterochromaffin cells, a subtype of EEC, in the gut and 5% in the CNS (Gershon and Tack, 2007). So far, there is no evidence for the production of 5-HT

by the gut microbiota, but studies have shown that microbiota mediates 5-HT synthesis in EEC (Reigstad et al., 2015; Yano et al., 2015).

No evidence supports that 5-HT produced in the gut can cross the BBB. Nakatani et al., 2008 showed that brain-derived 5-HT could cross the BBB to reach the peripherical circulation in rats. Interestingly, microbes in the gut have been shown to influence the brain 5-HT level in a mouse model (Clarke et al., 2013). The authors showed that the concentration of 5-HT in the hippocampus and Trp in plasma of the male GF mice were elevated compared with conventionally colonised control mice, suggesting a peripheral circulatory route through which microbiota influences brain 5-HT levels (Clarke et al., 2013). More studies are required to evaluate the bi-directional transport of 5-HT across the BBB and the potential regulatory role of the gut microbiota. Recently, studies have shown that certain commensal microbes and probiotic strains can uptake luminal 5-HT via specific transporters, which in turn can influence the microbial colonisation of the gut (Lyte and Brown, 2018; Fung et al., 2019). Together these findings indicate that the gut microbiota requires 5-HT to be produced in the gut and regulates the concentration of 5-HT in the gut and brain. Hence, the role of microbiota in the host serotonergic system warrants further attention.

Trp is metabolised to 5-HT in a two-step process (Figure 2-5). Trp hydroxylase (TPH), a rate-limiting enzyme in the biosynthesis of 5-HT, exists in two isoforms: TPH1 in the gut and TPH2 in the brain (Bender, 1983; Badawy, 2019). The conversion of 5-HT to melatonin is another two-step process and is catalysed by two limiting enzymes: N-acetyl transferase and hydroxyindole-O-methyltransferase (Bender, 1983; Zagajewski et al., 2012). Melatonin is produced in the gut mucosa and the pineal gland (Zagajewski et al., 2012). Melatonin regulates circadian rhythms of behaviour, physiology, sleep patterns and gut motility (Richard et al., 2009).

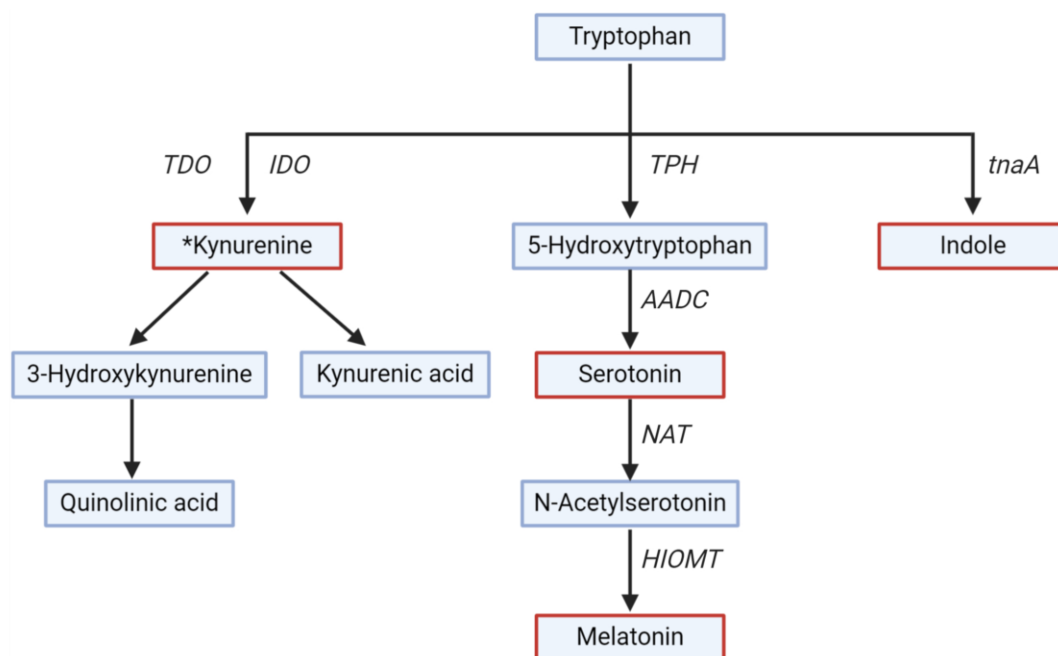



**Figure 2-5 Tryptophan metabolism along different pathways.**

Key metabolites (serotonin, melatonin, kynurenine, and indole) are in red. Rate-limiting enzymes shown are Trpytophan-2,3-dioxygenase (TDO), indoleamine-2,3-dioxygenase (IDO), tryptophan hydroxylase (TPH), Aromatic L-amino acid decarboxylase (AADC), N-acetyl transferase (NAT), hydroxyindole-O-methyl transferase (HIOMT), tryptophanase (tnaA).*All the downstream metabolites and enzymes of the kynurenine pathway are not shown for simplicity.

The kynurenine pathway is gaining interest due to the role of kynurenine and downstream metabolites (collectively called kynurenines) on the gut and brain functions and, thus, on GBA signalling (Cervenka et al., 2017). The kynurenine pathway accounts for around 90% of Trp degradation (Badawy, 2017). Kynurenine is produced from Trp by the action of Trp-2,3-dioxygenase and indoleamine-2,3-dioxygenase (IDO) (Platten et al., 2019). Kynurenine is further metabolised into downstream metabolites, of which kynurenic acid, 3-hydroxykynurenine, and quinolinic acid are important for their neuroactive effect on the brain (Badawy, 2017). The metabolite kynurenic acid has a neuroprotective effect, while 3-hydroxykynurenine and quinolinic acid have a neurotoxic effect (Schwarcz and

Stone, 2017). The enzyme Trp-2,3-dioxygenase is expressed in the liver, and IDO is widespread in numerous tissues, including the gut and the brain (Le Floc'h et al., 2011). The activity of IDO is regulated by proinflammatory cytokines (e.g., interferon-γ) released by toll-like receptor activation (Mahanonda et al., 2007), suggesting that the kynurenine pathway is more active in periods of immune activation or pathological conditions (Clarke et al., 2012). Unlike 5-HT, kynurenine and 3-hydroxykynurenine produced in the gut can cross the BBB and be further metabolised in the brain (Fukui et al., 1991).

An increase in Trp metabolism along the kynurenine pathway can result in a reduced availability of Trp for 5-HT synthesis and increased production of harmful kynurenine metabolites in the brain, contributing to mood disorders (Maes et al., 2011). In addition, this increased Trp metabolism may also imply decreased melatonin levels which are associated with circadian malfunctioning and can increase the risk of mood disorders (Quera-Salva et al., 2011). Interestingly, melatonin appears to promote the expression of IDO, suggesting a negative feedback loop through which melatonin regulates the balance between kynurenine and 5-HT pathways (Li et al., 2017).

The gut microbes can also metabolise Trp (Wikoff et al., 2009; Zheng et al., 2011; Waclawiková and El Aidy, 2018). The primary metabolite produced by the microbial metabolism of Trp is indole, which is catalysed by the enzyme tryptophanase (Jaglin et al., 2018; Waclawiková and El Aidy, 2018). Recently, Jaglin et al., 2018, have shown that administration of indole directly in the rat's cecum, where microbes metabolising Trp to indole are highly abundant, was associated with decreased motor activity and anxiety-like behaviour. However, the effect of indole on the human brain and behaviour has not been studied yet.

It is important to note that Trp metabolites, kynurenine, kynurenic acid, indole, and indole-derivatives, are important ligands for aryl hydrocarbon receptor (AHR) (DiNatale et al., 2010; Mezrich et al., 2010; Jin et al., 2014). The AHR is a cytoplasmic ligand-induced receptor, which is ubiquitously expressed in almost all tissues (Yamamoto et al., 2004) and contributes to immune homeostasis by having antimicrobial and anti-inflammatory effects (Zelante et al., 2013, 2014). For instance, lactobacilli utilise Trp to produce indole-3-aldehyde, an AHR ligand, activating innate lymphoid cells that provide mucosal resistance against the pathogen *Candida albicans* (Zelante et al., 2013). Interestingly, microbial metabolites such as SCFA regulate AHR and its target genes in the gut, likely influencing the microbial composition (Korecka et al., 2016). However, evidence of the role of AHR in brain development and function is limited. A study by Latchney et al., 2013 showed altered hippocampus neurogenesis and contextual fear memory in AHR-deficient adult mice, suggesting the role of AHR in brain development. However, whether the regulation of neurodevelopment by AHR is due to Trp metabolites is yet to be proven.

The combined increase in surface area for nutrient absorption (Thompson et al., 1998) and diversity of the commensal microbiota (Nagpal et al., 2017) during the maturation of the gut in the early postnatal period means that more Trp is absorbed and/or more Trp metabolites are produced and released in the peripheral circulation. However, the impact of gut maturation on Trp metabolism in early postnatal life is poorly understood. Interestingly, a study in infants showed that cereals enriched with Trp increased plasma concentrations of melatonin and improved sleep quality (Cubero et al., 2009). As the sleep-wake cycle is controlled by Trp-derived melatonin (Brown, 1994), and more melatonin levels result in better sleep (Cubero et al., 2009). This evidence could indicate more Trp metabolism through the hydroxylation pathway than other pathways. The role

of the Trp pathways and resulting neuroactive metabolites in brain development and function in early postnatal life is a fertile area of research.

### 2.4.4.2 Short-chain fatty acids

SCFA are saturated fatty acids with a chain length of one to six carbon atoms. They are the primary end-products of microbial fermentation and are produced in the gut depending on the content of dietary (e.g., fibre) (Bergman, 1990) and non-dietary components (e.g., mucins) (Hoskins and Boulding, 1981; Montoya et al., 2017). The most abundant SCFA produced in the human gut lumen are acetate, butyrate, and propionate (Dalile et al., 2019). Most SCFA produced, are absorbed (Ruppin et al., 1980; Hoogeveen et al., 2020) and utilised by enterocytes as an energy source at different ratios (Huda-Faujan et al., 2010; Dalile et al., 2019). Acetate the most abundant SCFA, and is produced by many microbes, while butyrate and propionate are produced by fewer gut microbial species (Cummings et al., 1987; Morrison and Preston, 2016).

The SCFA regulate various gut functions. For instance, butyrate, acetate, and propionate help to maintain barrier integrity, protect from inflammation and affect mucous production in the gut (Dalile et al., 2019). Recently, SCFA have been gaining attention for their potential role in the GBA. Studies have found that GLP-1- and PYY-secreting EEC, co-expressed SCFA receptors like free fatty acid receptors 2 and 3 (Karaki et al., 2008; Tolhurst et al., 2012), and deletion of these SCFA receptors in EEC in a mouse model has resulted in impaired PYY expression (Samuel et al., 2008) and reduced GLP-1 blood concentration (Tolhurst et al., 2012). These findings suggest that SCFA may stimulate the release of these gut hormones that act as essential mediators of GBA function, as discussed above. In addition, SCFA have been shown to promote TPH1 expression in a human carcinoid cell line derived from pancreatic tissues that share functional similarities with EEC, suggesting that SCFA can regulate the production of 5-

HT by EEC (Reigstad et al., 2015). However, caution must be exercised while translating cell line results to humans, as these cells divide continuously and may express unique gene patterns absent in cells *in vivo* (Kaur and Dufour, 2012). Further evidence of the importance of SCFA in the GBA comes from a study where butyrate administration by intraperitoneal injection has been shown to attenuate social behaviour deficiency in rodents (Kratsman et al., 2016). Butyrate and propionate can also activate tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis (c.f., Section 2.4.4.3) (Nankova et al., 2014).

Other studies showed that SCFA could also directly influence the GBA. Brain uptake of SCFA was reported following the injection of a mix of $^{14}$C-SCFA into the carotid artery, which suggests that BBB might be permeable to SCFA (Oldendorf, 1973). However, whether the concentration of organic acids injected was physiologically relevant remains to be elucidated. SCFA might also directly activate vagal afferents. Luminal perfusion of sodium butyrate into the jejunum of anesthetised male rats evoked vagal efferent nerve responses that were abolished following vagotomy (Lal et al., 2001). Therefore, SCFA can participate in GBA directly and indirectly; however, further studies are required to understand their role in GBA under physiological conditions.

In the early postnatal period, SCFA production and proportion are expected to change in response to microbial colonisation of the gut (Midtvedt and Midtvedt, 1992; Norin et al., 2004; Bergström et al., 2014). For instance, exclusively breastfed infants had relatively more acetate in their faeces as compared to non-breastfed infants (Bridgman et al., 2017), likely due to the fermentation of oligosaccharides present in human breast milk by members of the *Bifidobacterium* genus (Azad et al., 2016). The introduction of solid food results in the establishment of different microbial colonisers, which change the SCFA profile in the faecal sample (Differding et al., 2020). However, the direct and indirect

effects of SCFA production in the early postnatal period on GBA and subsequent consequences for developing the brain and behaviours are poorly understood.

#### 2.4.4.3 Neurotransmitters

Chemical substances that carry information between neurons are called neurotransmitters. There are about 100 different neurotransmitters produced in the body, each with different functions. Based on chemical composition, neurotransmitters are mainly classified as amino acids and biogenic amines. Functionally, neurotransmitters can be classified as excitatory (increase action potential firing), inhibitory (decrease action potential firing), or modulatory (fine-tune the action of both excitatory and inhibitory neurotransmitters).

Dietary amino acids are precursors for synthesising 5-HT, GABA, norepinephrine, dopamine, and histamine. For example, the synthesis of 5-HT is exclusively from dietary Trp. In contrast, dietary phenylalanine (an essential amino acid) serves as a precursor to tyrosine (a non-essential amino acid), which is essential for the synthesis of norepinephrine and dopamine, and histidine (an essential amino acid) serves as a precursor for histamine (reviewed in (Fabisiak et al., 2017; Mittal et al., 2017; Fernstrom and Fernstrom, 2018).

Genes responsible for metabolising amino acids to neurotransmitters (or precursors of thereof) have been identified in some bacteria *in vitro*. For instance, *Lactobacillus* and *Klebsiella* spp. possess a histidine decarboxylase gene that converts histidine to produce histamine (Kim et al., 2001; Lucas et al., 2008). *Legionella pneumophila* and *Pseudomonas* spp. have a phenylalanine hydroxylase gene that facilitates the conversion of phenylalanine to tyrosine (precursor of dopamine and norepinephrine), which has been demonstrated *in vitro* (Letendre et al., 1975; Flydal et al., 2012). From the above evidence, it could be speculated that neurotransmitter production by the gut microbes

might be modulated by dietary amino acids and contributes to GBA signalling. A list of neurotransmitters and their production by microbial species and amino acid precursors is shown in Table 2-1.

**Table 2-1 Potential neurotransmitters in the gut-brain axis**

| Neurotransmitter | Amino acid precursor | [1]Microbial species | Gut role | Brain role |
|---|---|---|---|---|
| Serotonin | Tryptophan | *Escherchia coli* (K-12), *Klebsiella pneumoniae* (Özoğul, 2004; Shishov et al., 2009) | Regulates gastric secretion and motility (Misiewicz et al., 1966) | Mood regulation by decreasing anxiety and stress (Williams et al., 2006) |
| [2]GABA | [3]Glutamine | *Lactobacillus brevis* and *Bifidobacterium dentium* (Barrett et al., 2012) | Regulates gastric emptying, secretion, and motility (Hyland and Cryan, 2010) | Processes sensory information and regulates memory and anxiety (Kalueff and Nutt, 1996) |
| Dopamine | Phenylalanine | *Escherichia* and lactic acid-producing bacteria such as *Lactococcus* and *Lactobacillus* spp. (Shishov et al., 2009; Özogul, 2011) | Regulates motility (Li, 2006) | Voluntary movement induces a feeling of pleasure (Juárez Olguín et al., 2016) |
| Norepinephrine | Phenylalanine | *Escherichia, Bacillus* and *Saccharomyces* spp. (Shishov et al., 2009; Lyte, 2011) | Regulates blood flow (Schwarz et al., 2001) | Motor control, emotion and endocrine modulation (Kobayashi, 2001) |
| Histamine | Histidine | *Lactobacillus* and *Pediococcus* spp. (Landete et al., 2007; Özoğul et al., 2012) | Modulates motility and enhances gastric acid production (Kano et al., 2004; Kim et al., 2011) | Regulates wakefulness, and motivation (Brown et al., 2001; Torrealba et al., 2012) |

*[1]The list of bacterial strains is mostly based on in vitro studies and may not be present in vivo and are provided as examples. [2]Gamma aminobutyric acid (GABA) is the only inhibitory amino acid neurotransmitter; all others are modulatory biogenic neurotransmitters. [3]Glutamine is the only non-essential amino acid precursor, whereas all other precursors of neurotransmitters are essential amino acids*

Some studies report evidence of the metabolism of neurotransmitters by the gut microbiota. For example, pathogenic *Escherichia coli* O157:H7 has an increasing growth rate in the presence of norepinephrine and dopamine (Freestone et al., 2002). In addition, an extract of the peel and pulp of a banana, which is rich in neurochemicals (e.g., norepinephrine, dopamine, 5-HT), has been shown to promote the growth of pathogenic and non-pathogenic bacteria (Lyte, 1997). However, the mechanisms by which the gut microbiota can metabolise neurotransmitters *in vivo* are yet to be understood.

There is accumulating evidence *in vivo* suggesting that the gut microbiota plays a role in modulating the abundance of neurotransmitters. For instance, GF mice have reduced levels of norepinephrine in caecal content (Asano et al., 2012) and GABA in faeces and plasma (Matsumoto et al., 2013). The turnover rate of norepinephrine, dopamine and 5-HT was higher in the striatum (part of the brain) of GF mice compared with the SPF mice (Heijtz et al., 2011). These reduced levels of neurotransmitters are in line with the altered anxiety-like response in the GF phenotype, suggesting the role of gut microbiota in modulating behaviour (Heijtz et al., 2011; Neufeld et al., 2011). However, no studies have yet reported whether the gut microbiota directly affects the level of neurotransmitters in the body or modulates the host production of neurotransmitters.

There is also no evidence of whether neurotransmitters from the gut can cross the BBB to reach the brain. Interestingly, the vagal afferent nerve expresses receptors for 5-HT, GABA, and dopamine (Egerod et al., 2018), suggesting the possibility of an alternative route for communication between the gut and the brain. Therefore, gut-derived neurotransmitters appear to be a potential mediator of the GBA, and further studies are required to confirm their potential.

In the early postnatal period, histological (e.g., crypt depth) and functional (e.g., enzyme) gut changes can result in different rates of amino acid uptake and host neurotransmitter production. The increased relative abundance and diversity of the gut microbiota could also influence neurotransmitter production. For instance, *Bifidobacterium* strains have been shown to dominate the faecal microbiota of breastfed infants (Kato et al., 2017; Nagpal et al., 2017; Lawson et al., 2020) and also one of the strains *Bifidobacterium brevis* has shown the ability to produce GABA (Barrett et al., 2012). Change in the abundance of different *Bifidobacterium* strains postnatally (Kato et al., 2017) could alter the GABA level in the gut. Changes in the production of neurotransmitters (type and amount) and their role in the GBA in response to early postnatal developmental remain to be established.

## 2.5 Early nutrition during the critical window of development

Nutrition in early life (conception to first years of postnatal life) is one of the crucial determinants of developmental outcomes in adulthood. Prenatally, maternal nutrition plays an essential role in foetal growth and development. Postnatally, human breast milk offers nutrition and other benefits, including modulation of gut and brain development and function. Breast milk is the best source of human nutrition, but when breast milk is unavailable or limited, industrially produced substitutes (formula) based on milk from ruminant species, mimicking the nutritional composition of breast milk, are offered to infants.

### 2.5.1 Human breast milk

Human breast milk is a complex matrix containing carbohydrates, lipids, proteins, vitamins, minerals, growth factors, enzymes, and bioactive molecules. Human breast milk composition varies between feeding sessions, stages of lactation and between mothers.

During feeding sessions, the consistency of foremilk is thinner with higher lactose content, followed by hindmilk with a creamier consistency and higher fat content than foremilk (Martin et al., 2016). During the stages of lactation, colostrum produced in the first few days of postpartum contains high quantities of immunoglobulins, lactoferrin, and growth factors and low quantities of lactose (Ballard and Morrow, 2013). Transitional milk is produced from day five to two weeks postpartum and contains a high amount of lactose, fat, and protein compared to colostrum (Ballard and Morrow, 2013). By four to six weeks postpartum, milk composition becomes more stable and compositionally mature and contains high lactose, water, and vitamins B1 and B6 than colostrum and transitional milk (Ballard and Morrow, 2013).

#### 2.5.1.1 Macro- and micronutrients

Triglycerides (TG) are the main lipid fraction of human breast milk, accounting for about 98% of total milk lipids (Koletzko, 2017). The remaining lipids in the milk include monoglycerides, diglycerides (DG), phospholipids, free fatty acids, and cholesterol (Koletzko, 2017). Nearly 50% of milk fatty acids are saturated, with 25% being palmitic acid. Monounsaturated fatty acids (MUFA) account for 40-45% of breast milk, with 36% being oleic acid (Martin et al., 2016; Koletzko, 2017). LCPUFA accounts for 15% of breast milk, with a major portion (~14 %) of linoleic acid (Ramiro-Cortijo et al., 2020). An *in vitro* study showed that fermentation of human breast milk MUFA, monoacylglycerol, and/or sphingosine using an infant faecal inoculum increased *Lactobacillus* and *Bifidobacterium* spp. and decreased *Enterobacteriaceae family* abundances (Nejrup et al., 2015). Another study has shown that early enteral supplementation of LCPUFA increased the ileal villus height and muscular thickness in piglets (Akinsulire et al., 2020). Milk lipids were also found to influence brain development. A study has shown that higher levels of human breast milk SM were

associated with increased myelin content and higher verbal development in the first two years of development (Schneider et al., 2019).

Proteins of human breast milk are divided into two fractions: casein and whey. Casein fraction includes α-, β-, and κ-casein. Whey fraction includes lactoferrin, α-lactalbumin, secretory immunoglobulin IgA and lysozyme. Approximately 25% of the total nitrogen of human breast milk comes from non-protein nitrogen compounds, including urea, creatine, uric acid, creatine, creatinine, amino acids, and nucleotides. A study has shown that preterm piglets receiving lactalbumin-enriched whey protein concentrate for 19 days increased the colonic microbiota diversity and relative abundances of the families *Clostridiaceae, Enterobacteriaceae* and genera *Streptococcus*, and *Streptomyces* (Nielsen et al., 2020). Additionally, the amino acid Trp the precursor of 5-HT, can cross the BBB to influence brain development (as described in Section 2.4.4).

Lactose is the principal carbohydrate of human breast milk. It represents 30% to 40% of human breast milk energy content. The effects of lactose on gut and brain development are not well understood. An *in vitro* study showed lactose and bovine milk oligosaccharides synergistically enhance the growth of *Bifidobacterium longum* using mono and co-cocultures of bacteria present in the infant's gut (Jakobsen et al., 2019).

Human breast milk contains adequate amounts of micronutrients to support the growth of infants. Vitamin A, vitamin D and minerals (e.g., iron, zinc, iodine, and calcium) are important micronutrients in human breast milk. In addition, micronutrients play an important role in gut and brain development and function. For instance, studies in rodents have shown that vitamin D deficiency by dietary restriction altered the epithelial barrier dysfunction and composition of the gut microbiota (Ooi et al., 2013; Assa et al., 2014). Additionally, in the brain, choline is a precursor for acetylcholine neurotransmitters, PC

and SM, and a study has shown that a deficiency of choline leads to memory deficits (Zeisel, 2006).

#### 2.5.1.2 Bioactive components

Human breast milk contains several non-nutritive components to support physiological functions. These components are bioactive components of human milk. Excellent reviews are available on human milk bioactive components (Ballard and Morrow, 2013; Gila-Diaz et al., 2019). This review will focus on the most abundant bioactive components of human breast milk; HMO and milk fat globule membranes (MFGM), and their role in early postnatal life development.

HMO are one of the major components of human breast milk and made up of five basic units: sialic acid, N-acetylglucosamine, L-fructose, D-galactose, and D-glucose (Wiciński et al., 2020; Zhang et al., 2021). So far, 200 different types of HMO have been characterised in human breast milk based on the different configurations of the five basic units (Zhang et al., 2021). Additionally, the HMO core structure consists of a lactose core which is further lengthened by type1 chain (lactose-N-biose) or type 2 chain (N-acetyllactosamine) and can also be sialylated and/or fucosylated (Zhang et al., 2021). Hence, based on the structure, HMO are classified into three groups, namely: fucosylated, sialylated, and neutral HMO (Zhang et al., 2021).

HMO cannot be digested by mammalian digestive enzymes and are indigestible carbohydrates that are fermented by the specific microbes of the large intestine (e.g., *Bifidobacterium spp.*, *Bacteroides* spp., and *Lactobacilli*) (Bunesova et al., 2016; Garrido et al., 2016; Thongaram et al., 2017). HMO play a crucial role in establishing the infant gut microbiota (Coppa et al., 2011; Lewis et al., 2015), inducing epithelial cells maturation and improving gut barrier function (Akbari et al., 2017). Brain development

is also influenced by HMO. A study has shown that consuming HMO, 2'-fucosyllactose at 24 months of age was linked with cognitive development related to learning and memory functions (Berger et al., 2020). Another study has shown that piglets fed a milk diet supplemented with sialyllactose enhanced spatial cognition and increased the expression of hippocampal genes associated with sialic acid metabolism, myelination, and ganglioside biosynthesis (Obelitz-Ryom et al., 2019).

MFGM is a tri-layer membrane complex surrounding a triglyceride core, the milk fat globule. The constituents of MFGM include but are not limited to phospholipids, glycoproteins, glycolipids, fatty acids, and proteins. Studies in animal models have shown that supplementation of infant formula with MFGM promoted microbial colonisation and intestinal barrier function in early postnatal life (Bhinder et al., 2017; Gong et al., 2020; Wu et al., 2021). Studies have also shown the role of MFGM in brain development (Brink and Lönnerdal, 2018; Moukarzel et al., 2018). For instance, SM, one of the components of MFGM, increased myelin production in rat pups (Oshida et al., 2003) and the neuro-behavioural development of low-birth-weight infants (Tanaka et al., 2013). Additionally, choline attached to the PC component of MFGM (Brink and Lönnerdal, 2020) acts as a precursor for the acetylcholine neurotransmitter crucial in regulating learning and memory functions (Hasselmo, 2006).

### 2.5.2 Infant formula

Infant formula is an effective substitute for human breast milk when breast milk is unavailable or limited. The infant formula manufacturing process is highly regulated and aims to mimic the nutrient composition, bioactive composition, taste, and texture of breast milk.

Ruminant milk is the most common base for infant formula, with bovine milk most widely used, while other ruminant species, ovine and caprine milk, are steadily gaining use in the infant formula industries. Bovine, ovine, and caprine milk are different in composition. Ovine milk has more proteins, fats, and total solids than caprine and bovine milk (Claeys et al., 2014). Caprine milk has a higher content and variety of oligosaccharides (Martinez-Ferez et al., 2006) and a lower content of lactose than bovine and ovine milk (Claeys et al., 2014). Ovine milk has a higher content of essential and non-essential amino acids (except for glycine and cysteine) than bovine and caprine milk (Claeys et al., 2014; Rafiq et al., 2016). Bovine milk has higher saturated fatty acid content than ovine and caprine milk (Devle et al., 2012). In contrast, ovine milk has more MUFA and polyunsaturated fatty acids (PUFA) than bovine and caprine milk (Devle et al., 2012). Reviews of the detailed information on the comparative composition of ruminants' milk have been published elsewhere (Barlowska et al., 2011; Claeys et al., 2014; Gantner et al., 2015).

Different ruminant's milk imparts different physiological responses in the host. For example, a study has shown that the gastric emptying rate of milk proteins and fat in piglets varies between ovine, caprine, and ovine species (Roy et al., 2022a). The authors suggested that this observation was due to milk structural variation between the ruminant species, which has been extensively reviewed by Roy et al., 2020. *In vitro* studies have also shown that fermentation of predigested ruminants' milk using a faecal inoculum of infants showed that the microbial composition of fermentation samples differs between them (Ahlborn et al., 2020; Gallier et al., 2020). The effects of different ruminant's milk on neurodevelopment and function have not been studied yet. However, it could be hypothesised that consuming different ruminant milk in early postnatal life could influence gut development differently, influencing brain development via the GBA (c.f.,

section 2.4). However, no studies have yet been conducted to understand the effects of ruminants' milk on early postnatal development.

## 2.6 Summary

The early postnatal years of life are marked by rapid developmental changes in the gut and brain. Gut tissue, ENS and microbial maturation coincide with the refinement and remodelling of brain neural circuits and cognitive development in the first years of life. Sophisticated complex communication systems involving mediators such as the VN, gut hormones, cytokines, and gut-derived metabolites are known to govern the crosstalk between the gut and the brain.

Early life foods (breast milk, formula, and complementary foods) play a crucial role in the development of the gut and brain via nutritional programming and modulation of the GBA in the early postnatal years of life. Therefore, understanding better the connection between early nutrition, gut development and brain development is important to optimise early postnatal brain development and behavioural outcomes via dietary interventions.

Although existing evidence has pointed towards the role of breast milk in early postnatal life brain development via the GBA, studies on the role of milk from other species that are used to make infant formula remain elusive. Furthermore, most of the studies on perinatal development have been carried out in rodent animal models, but considerable differences in developmental patterns of the gut and the brain between humans and rodents exist. Therefore, the use of animal models with more comparable anatomy and physiology (e.g., piglets, primates) to that of humans is desirable to gain a better understanding of the role of nutrition in postnatal early life development and improve the translation of research to infants.

## 2.7 Research questions

This project is a part of the MBIE Smarter Lives programme, which seeks to investigate how the intake of dairy and/or food ingredients may influence cognition, memory, and stress/anxiety resilience via the gut in young adults and ageing humans. But due to the COVID-19 associated lockdowns and delays, samples were obtained from another programme (MBIE, New Zealand Milk Means More), which aims to provide a mechanistic understanding of differences in the outcomes arising due to variation between ruminants' milk. Integrating both MBIE programme goals, this thesis aimed to understand the effects of different ruminants' milk on early postnatal brain development via gut-derived metabolites in piglets.

Specifically, this thesis sought to address the following research questions:

**RQ1**- Does the circulatory blood plasma metabolite profile differ between bovine, caprine and ovine milk treatments during the early postnatal period?

**RQ2**- Does the metabolite profile of the brain areas crucial for cognitive development differ between bovine, caprine and ovine milk treatments during the early postnatal period?

**RQ3**- Does the expression of genes in the areas of the brain crucial for cognitive development differ between bovine, caprine and ovine milk treatments during the early postnatal period?

**RQ4**- If the metabolite profile in plasma and metabolite and gene expression profile in brain areas change in response to different ruminant milk treatments, do these changes correlate?

## 2.8 Research thesis structure

Chapters 3, 4, 5 and 6 are the main research chapters and present the results from analyses of the samples collected from the animal study conducted by Dr Debashree Roy as a part of her doctoral study, focussing on structural changes in bovine and non-bovine (ovine and caprine) whole milk during digestion of piglets in early postnatal life.

Prior research has highlighted the importance of gut-derived metabolites in the signalling between the gut and the brain in the early postnatal period. However, there are no comprehensive analyses of all the plasma metabolites (polar and non-polar) detected in peripheral blood in response to bovine, ovine or caprine treatments in this period. Chapter 3, therefore, explores the effects of whole milk from bovine, caprine and ovine species on the circulatory blood plasma metabolome using a liquid chromatography-mass spectrometry (LC-MS) approach. Peripheral blood plasma metabolites were analysed as they are influenced by changes in the gut (tissue and microbiota) in the early postnatal period.

Metabolites are considered a crucial determinant of brain functional development. They play a role in cell signalling, neural membrane structure, receptor membrane protein functions, synaptic plasticity, and memory formation, However, there is no data on the global profile of metabolites (polar and non-polar) in the brain regions associated with cognitive development in response to bovine, ovine or caprine milk treatment in the early postnatal period. Chapter 4, therefore, explores the effects of whole milk from bovine, caprine and ovine species on the metabolome of the hippocampus, prefrontal cortex, and striatum using a LC-MS approach.

Functional development of the brain can be assessed through protein expression, functional neuroimaging, and behavioural studies. Understanding the underlying

molecular features is a step towards understanding brain functional development. However, the gene expression patterns associated with cognitive development in these specific brain areas in response to ruminant milk treatments have not been previously evaluated. Chapter 5, therefore, explores the effects of whole milk from bovine, caprine and ovine species on the expression of selected neurotransmission, learning and memory genes in the same brain areas as Chapter 4, using NanoString technology.

The interaction between diet, metabolites found in the peripheral blood circulation and the brain has been of particular interest in early postnatal brain development. However, no studies have explored these interactions in the context of ruminants' milk. Chapter 6 explores the correlations between peripheral metabolite relative intensity (Chapter 3), brain metabolite relative intensity (Chapter 4) and brain gene expression (Chapter 5) using the multi-omics data integration method.

The thesis concludes with a general discussion (Chapter 7) that brings together the main results of Chapters 3, 4, 5 and 6. The discussion highlights the significance and relevance of these results, the strengths and limitations of the thesis research and the consideration of future research.

# Chapter 3

# Plasma metabolite profiling of peripheral blood in response to ruminants' milk in early postnatal life

# Abstract

Ruminant milk is commonly used for supplying nutrients to infants when breast milk is unavailable or limited. Previous studies have highlighted the differences between ruminants' milk composition, digestion, absorption, and fermentation. However, whether consuming different ruminants' milk impact the appearance of the circulatory blood metabolites in early postnatal life is not well understood. The analysis conducted here aimed to determine the effect of feeding exclusively whole milk from bovine, caprine or ovine species to piglets, approximately seven days old for fifteen days, on circulatory blood plasma metabolites. Relative intensities of plasma metabolites were detected using an LC-MS metabolomics approach.

Seven polar and 83 non-polar (lipids) metabolites in plasma were significantly different (false discovery rate (FDR) corrected P-value < 0.05) between milk treatments. These included polar metabolites involved in amino acid metabolism and lipids belonging to PC, lysophosphatidylcholine (LPC), SM, TG, and DG. Compared to the caprine or bovine milk group, the relative intensities of polar metabolites and unsaturated TG increased in the peripheral circulation of the ovine milk group.

In contrast, relative intensities of saturated TG and PC were increased in the bovine milk group compared to the ovine or caprine milk group. In addition, correlations were identified between amino acid and lipid intakes and their appearances in peripheral blood circulation. The results highlighted that consuming different ruminants' milk influences the plasma appearance of metabolites, especially lipids, that may contribute to early postnatal life development in piglets.

## 3.1 Introduction

During the early postnatal years of life, body development occurs rapidly, involving events like the maturation of the tissular structure and function of the gut, the establishment of gut microbiota, remodelling of the immune system, refinement of brain neural circuits, and establishment of cognitive abilities. Human breast milk or infant formula is one of the factors influencing the developmental events during this period. Human milk is the best nutrient source for infant development. However, formula feeding is crucial in satisfying nutrient requirements when breastfeeding is limited or unavailable.

Infant formula is predominantly made with bovine milk. However, non-bovine milk (e.g., caprine and ovine) is also increasingly used due to its hypo-allergenic properties and easier digestion than bovine milk (Park, 1994; Martinez-Ferez et al., 2006; Masoodi and Shafi, 2010; Crowley et al., 2017).

Nutrient composition (Park et al., 2007; Claeys et al., 2014; Rafiq et al., 2016; Felice et al., 2021) and physiochemical properties (Claeys et al., 2014; Crowley et al., 2017) between bovine, caprine, and ovine milk differ. Ovine milk has a higher concentration of macronutrients (lipids and proteins) and micronutrients (Ca, P) than bovine or caprine milk (Barlowska et al., 2011; Claeys et al., 2014). Additionally, caprine and ovine casein micelle diameters are greater than those in bovine milk, whereas fat globule diameters are less than those in bovine milk (Claeys et al., 2014; Crowley et al., 2017). Milk composition within the same species also varies considerably due to milking season, breed, type of feed and climate (Haenlein, 2004; Claeys et al., 2014).

Milk is largely digested in the stomach and small intestine. The released nutrients are absorbed in the small intestine and metabolised in host tissues. A piglet study by Roy et al., 2022a showed that the gastric emptying rate of proteins and lipids differed across

bovine, caprine and ovine milk, suggesting that this might lead to different absorption in the small intestine and, therefore, the appearance of these nutrients in the peripheral blood circulation across milk treatments.

Milk also contains nutrients like oligosaccharides known to escape digestion in the small intestine and used by the colonic gut microbiota (Brand-Miller et al., 1998; Gareau et al., 2010; Donovan et al., 2012). Digested ruminant milk that were dialysed to remove digested molecules and then the undigested molecules (retained after dialysis) were added as a substrate to batch cultures using a faecal inoculum from infants differentially altered the cultured microbial composition depending on the type of milk (Ahlborn et al., 2020; Gallier et al., 2020). Fermentation of the remaining undigested material from bovine and ovine milk also resulted in different concentrations of microbial metabolites (e.g., SCFA) between milk treatments (Ahlborn et al., 2020). Thus, it could be expected that host and microbial activities related to the consumption of milk from different ruminant species in early postnatal life might lead to the production of different metabolites involved in signalling, energy conversion, neurotransmission, and cofactor activity, which could influence the development of the first years of postnatal life. However, studies are yet to address whether consuming different ruminant milk in early postnatal life can modulate the appearance of metabolites in the peripheral circulation.

Based on the existing evidence, it was hypothesised that bovine, caprine or ovine milk with different nutrient compositions and gastric emptying rates would lead to a varied abundance of host and microbial metabolites appearing in the peripheral blood circulation. Therefore, the aim was to determine the effects of these milk treatments on the circulatory blood plasma metabolome in piglets as a model of human infants. In

addition, the amino acid concentration was quantified in the blood plasma and milk. Furthermore, milk lipid relative intensities were evaluated.

## 3.2 Materials and methods

### 3.2.1 Milk nutrient analysis

Three batches of raw milk samples collected during the study were stored at -20 °C before being freeze-dried and ground. The freeze-dried raw whole milk batches were analysed for standard amino acids using AOAC method 994.12, including HCL acid hydrolysis, followed by reversed-phase high-performance liquid chromatography (RP-HPLC). Cysteine and methionine were analysed using the AOAC method 994.12, including performic acid oxidation. Trp was analysed using alkaline hydrolysis, followed by RP-HPLC (Hugli and Moore, 1972; Ravindran and Bryden, 2005). Finally, freeze-dried milk samples were reconstituted with Milli-Q® water for lipid analyses using LC-MS lipidomics.

### 3.2.2 Animal study

The protocol used for the piglet study was approved by the Massey University Animal Ethics Committee (MUAEC protocol 18/97) and described in detail elsewhere (Roy et al., 2022a). Twenty-four male piglets (~postnatal day (PND) 7, mean body weight (BW) on arrival 3 kg (1.9 to 3.5)) were obtained from a local commercial farm (Aorere Farms Partnership, Whanganui, New Zealand). Information on the number of litters these piglets were obtained was unavailable. These animals were housed in purpose-built plastic metabolism crates (700 x 450 x 500 mm) in a room with a 16 h light-8 h dark cycle and a temperature of 28 ± 2 °C. The crates of these animals were enriched with toys, which

were replaced with clean toys daily. Piglets were allowed to socialise every day under supervision for an hour.

Upon arrival, these piglets were randomly assigned to three diet groups (bovine, caprine, or ovine milk), such that there were eight piglets per milk treatment. From birth to ~PND 6 (the period before the experimental day), these piglets consumed *ad libitum* sow's milk. The experimental period comprises ~PND 7 to ~PND 21, where piglets were bottle-fed one of the milk treatments. From ~PND 7 to PND 18, piglets were bottle-fed either bovine, caprine, or ovine-reconstituted whole milk powder diets (purchased from Davis Food Ingredients, Dairy Goat Co-operative, and Spring Sheep Milk Co., respectively), including vitamin and mineral supplements (purchased from Nutritech International Ltd.). From PND 19, the piglets were fed either bovine, caprine or ovine fresh raw whole milk obtained under chilled conditions from the Massey University No. 4 Dairy Farm (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand), Phoenix Goats (Palmerston North, New Zealand), and Neer Enterprises Limited (Carterton, New Zealand), respectively. The fresh milk was provided for three days only due to a limited supply of fresh ovine and caprine milk.

The piglets received iso-caloric and iso-volumetric amounts of each diet on a BW (Appendix Table A1) basis (345 g of liquid meal per kg of BW per day) from ~PND 7 to ~PND 13, which was considered the acclimatisation period for the piglets to learn to drink from the bottle with a rubber teat. The diets were balanced for protein content from ~PND 14 to ~PND 21 (2 g per kg BW). From ~PND 14 to ~PND 18 (reconstituted powder), the piglets received equal amounts of protein (2 g of protein per kg of BW in every single meal) and equal volumes of diet (345 g of liquid meal per kg of BW per day). From ~PND 19 (fresh milk), the piglets received their respective milk volumes based on equal amounts

of protein per kg of BW. Balancing the protein intake between groups allowed the investigation of the effects of structural changes in ruminants' milk on raw whole milk gastric digestion, which was the study's primary objective (Roy et al., 2022a). On the last experimental day (~PND 21), piglets were euthanised at 210 min post-feeding to allow time for nutrient absorption. A diagram illustrating the study timeline is shown in Figure 3-1.

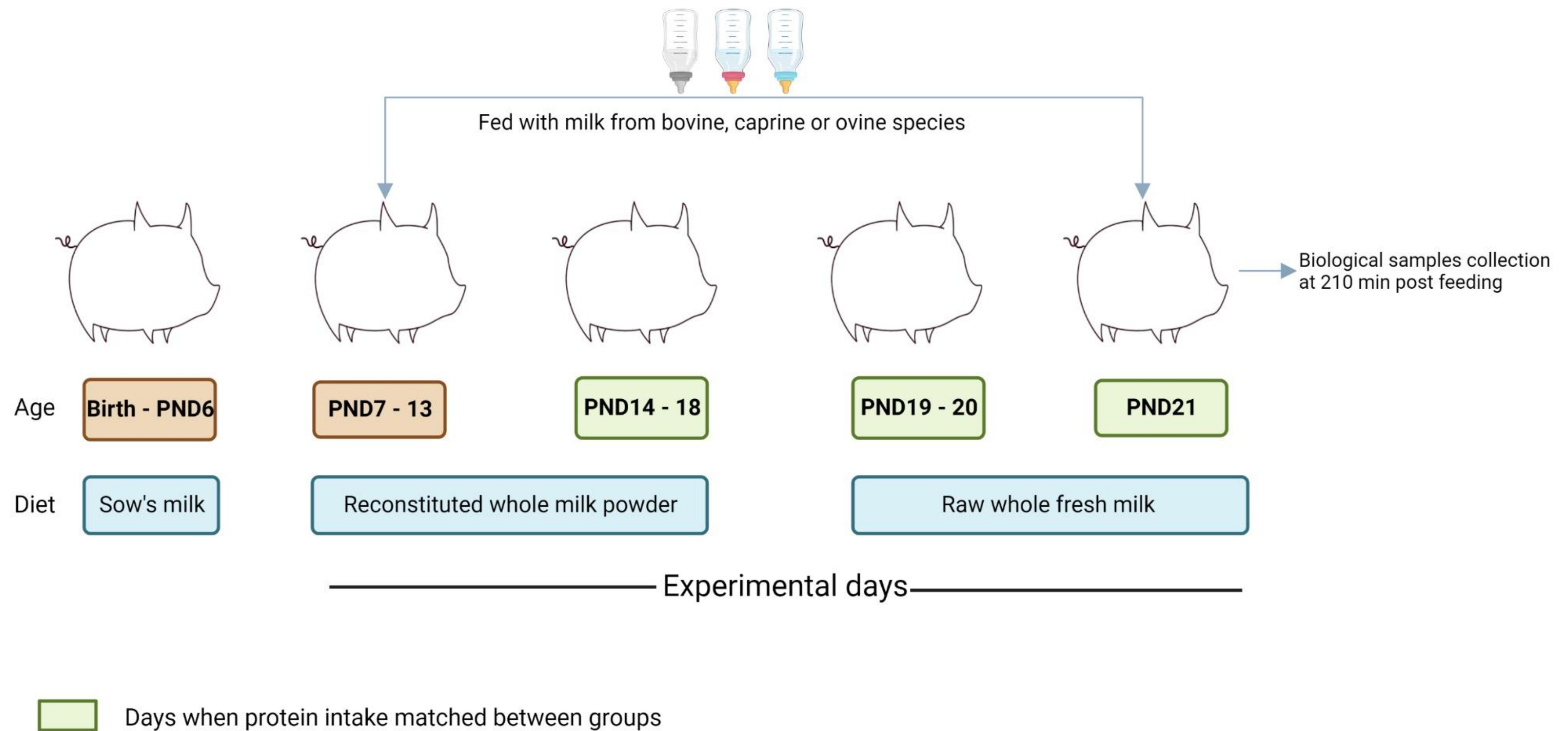



**Figure 3-1 Overview of the in vivo piglet study.**

PND, postnatal day. Figure created with biorender.com

### 3.2.3 Blood plasma sampling

The piglets were anaesthetised using Zoletil 100 (50 mg/mL of zolazepam and tiletamine each, Zoetis Inc., Parsippany-Troy Hills, NJ, USA) reconstituted with 2.5 mL each of ketamine and xylazine (both 100 mg/mL). Blood samples were drawn from the left ventricle and were collected in BD Vacutainer® blood collection tubes containing EDTA. Immediately after the collection, blood samples were centrifuged at 4500 rpm for 10 min at 4ºC, and the plasma was removed and stored at –80ºC until required.

### 3.2.4 Plasma amino acid analysis

The concentration of amino acids in plasma was analysed by AgResearch Analytical Laboratory (Palmerston North, New Zealand) using the Pico-Tag method (White et al., 1986) as described by (Milan et al., 2020). Briefly, 500 µL of each plasma sample was mixed with 10 µL of 10,000 µmol $mL^{-1}$ internal standard norleucine and 5 µL of 80,000 µmol $mL^{-1}$ dithiothreitol in 200,000 µmol $mL^{-1}$ phosphate buffer. The mixture was filtered using a 2mL Vivaspin 500 centrifugal concentrator and centrifuged for 90 min at 11,000 rpm at 4°C. Filtrate (50 µL) was lyophilised for 1 h in a freeze drier, followed by reconstitution in 20 µL freshly prepared methanol/1 M sodium acetate/triethylamine (2:2:1) and again lyophilised for 16 h. The lyophilised sample was reconstituted in freshly prepared methanol/Milli-Q water/triethylamine/phenylisothiocyanate (7:1:1:1) and then incubated for 20 min at room temperature. The reconstituted sample was then lyophilised for 2 h, followed by reconstitution in 0.71 mg $mL^{-1}$ disodium hydrogen orthophosphate in Milli-Q water with 10% phosphoric acid, diluted to 5% v/v in acetonitrile. Finally, the reconstituted sample was centrifuged for 5 min at 13,000 rpm at room temperature and the supernatant was collected for HPLC analysis.

Amino acids in the sample extracts were resolved on a PicoTag® column (60 Å, 4 µm, 300 mm x 3.9 mm) using an LC-10ADvp instrument (Shimadzu Corporation, Kyoto, Japan). Each sample extract of 50 µL were injected into a PicoTag® column, held at 46°C, and eluted over a 90-min gradient with a flow rate of 1 $mL \cdot min^{-1}$. The mobile phase used for chromatographic separation consisted of a mixture of buffer A [1,964 g of stock buffer (38.16 g sodium acetate trihydrate in Milli-Q water,10% v/v acetic acid), 35 g acetonitrile, and 500 µL 10 mM EDTA] and buffer B (900 mL acetonitrile, 800 mL Milli-Q water, and 300 mL methanol). Standards used for amino acids quantification were proteinogenic amino acid standard {consisting of AAS18 solution (2.5 µmol $mL^{-1}$ of 18 proteinogenic amino acids in 0.1 N HCl) and 0.05 µmol $mL^{-1}$ of L-glutamine, L-tryptophan, and L-asparagine}, and the composite physiological amino acid standard {consisting of equal volumes of A6282 (2.5 µmol $mL^{-1}$ of 14 physiological, basic amino acids in 0.1 N HCl) and A6407 (2.5 µmol $mL^{-1}$ of 26 physiological amino acids in 0.1 N HCl) solutions }. Amino acid quantification was based on the mean, standard response of each analyte.

### 3.2.5 Metabolomics analysis

#### 3.2.5.1 Chemicals

All chemicals and solvents used were LC-MS grade unless specified. Chloroform (analytical grade), methanol, acetonitrile, isopropanol, and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Milli-Q® ultrapure water was purchased from Merck Millipore (Bedford, MA, USA). Ammonium formate (HPLC grade) and internal standards ($d_5$-tryptophan, $d_{10}$-leucine, $d_2$-tyrosine, and $d_7$-alanine) used in extraction solvent were purchased from Sigma-Aldrich (St. Louis, MO, USA). SPLASH® lipidomix® MS standard was obtained from Avanti® (Alabaster, AL, USA),

which included all of the major lipid classes (15:0-18:1(d7) PC, 15:0-18:1(d7) PE, 15:0-18:1(d7) PS, 15:0-18:1(d7) PG, 15:0-18:1(d7) PI, 15:0-18:1(d7) PA, 18:1(d7) LPC, 18:1(d7) LPE, 18:1(d7) Chol Ester,18:1(d7) MG, 15:0-18:1(d7) DG, 15:0-18:1(d7)-15:0 TG, 18:1(d9) SM, cholesterol (d7)).

### 3.2.5.2 Sample preparation

Plasma samples were thawed overnight at 4°C and vortexed. Extraction solvent of 800 µL containing chloroform: methanol (1:1 v/v containing internal standards $d_5$- Trp, $d_{10}$-leucine, $d_2$-tyrosine, and $d_7$-alanine), precooled at -20°C was added to each 2 mL microcentrifuge tubes containing 100 µL of plasma. The mixture was vortexed for 30 s and then incubated for 60 min at -20°C. Then 400 µL of Milli-Q® water was added to each sample, vortexed for 30 s and centrifuged for 10 min at 11,000 rpm at 4°C. Subsequently, 200 µL aliquots of the supernatant and 250 µL of the bottom layer were transferred into new 2 mL microcentrifuge tubes for polar and non-polar metabolites analyses. The pooled polar quality control samples were prepared by combining 100 µL of the supernatant from each sample into a new tube, vortexed for 30 s and then aliquoted into multiple microcentrifuge tubes. Similar procedures were followed for non-polar quality control samples, except the aliquots of 80 µL were taken from the bottom layer. Blank samples were prepared using the above procedures, except the samples were replaced with 100 µL Milli-Q® water. All samples and blanks were dried under a stream of nitrogen at room temperature and stored at -80°C.

On the day of LC-MS analysis, the dried extracts were reconstituted in 300 µL of acetonitrile: water (1:1 v/v) containing formic acid (0.1 %) for polar metabolites and 800 µL of chloroform: methanol (2:1 v/v) for non-polar metabolites. The reconstituted polar metabolite mixture was vortexed for 15 s, then centrifuged for 10 min at 11,000 rpm at

4°C. Aliquots of 100 µL of polar extract and 200 µL of lipid extracts were transferred into a vial containing a volume insert. Then, 7 µL of SPLASH® lipidomix® was added to the insert containing the lipid extract only. The inserts containing the metabolite extracts were stored at 4°C for immediate metabolite analysis.

### 3.2.5.3 Liquid chromatography-mass spectrometry analysis

Metabolite extracts were analysed using an LC-MS-9030 coupled with a Nexera-x2 ultra performance liquid chromatography system (Shimadzu, Kyoto, Kyoto, Japan).

Chromatographic separation of polar metabolites was conducted at 30°C by injecting 5 µL of samples onto an Accucore™ hydrophilic interaction liquid chromatography (HILIC) column, 2.1 mm × 100 mm, 2.6 µm particle size (Thermo Fisher Scientific, Waltham, MA, USA), and with a mobile phase flow rate of 400 µL/min. The mobile phases consisted of a mixture of 10 mM ammonium formate in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B).

Chromatographic separation of non-polar metabolites was conducted at 60°C by injecting 2 µL of samples onto a CSH-C18 column, 2.1 mm × 100 mm, 1.7 µm particle size (Waters, Milford, MA, USA) and with a mobile phase flow rate of 400 µL/min. The mobile phases were a mixture of 10 mM of ammonium formate in water/acetonitrile/isopropanol (5:3:2 v/v) (solvent A) and 10 mM of ammonium formate in water/acetonitrile/isopropanol (1:9:90 v/v) (solvent B).

The mass spectral detection for polar metabolites was performed in positive and negative ionisation modes. In contrast, for non-polar metabolites, mass spectral detection was performed only in positive ionisation mode, as the positive mode captures most lipids. Full MS1 spectra for polar and non-polar metabolites were measured from 70 to 1000 m/z and 250 to 1250 m/z across the chromatogram. Data-independent acquisition was

carried out using 20 m/z windows covering 70 to 900 m/z mass ranges for polar metabolites and 300 to 1100 m/z mass ranges for non-polar metabolites. A cycle time of 0.6 sec, collision energy of 25 normalised collision energy units, a source voltage of +4.0 kV, interface, desolvation line, and heater block temperature of 300, 250, and 400 °C, and nebulising, heater, and drying gas (nitrogen) flow rates of 2, 10, and 10 L/min were used to acquired polar and non-polar data (Abshirini et al., 2021).

### 3.2.5.4 Data processing

Raw data files were converted to centroid mzML format using the Shimadzu file converter and were uploaded to MS-DIAL software (version 4.48) (Tsugawa et al., 2015) for subsequent data-processing steps, including peak detection, gap-filling, alignment, and noise removal. The processing parameters were kept as default except for minimum peak height and retention time tolerance. For polar metabolite data analysed in positive and negative ionisation modes, minimum peak heights were 1,000 and 1,500, respectively, and retention time tolerances were 0.15 and 0.3 min, respectively. For non-polar metabolite data analysed in positive ionisation mode, minimum peak heights were 1,000 and retention time tolerances were 0.15 min. MS data was acquired in data-independent acquisition mode to enable MS/MS spectral reconstruction. Data-independent acquisition MS/MS spectra were used for aligned peak identification. MS/MS public library containing 13,303 unique compounds (Tsugawa et al., 2015) and the built-in lipid library containing 257,000 *in silico* generated MS/MS lipid fragmentation spectra were used for polar and non-polar metabolite feature identification, respectively. Afterwards, data normalisation was conducted using the quality control samples (LOESS normalisation) and the features with a quality control coefficient of variation > 30% were removed. Identified missing values were treated with the k-nearest neighbour method using Metaboanalyst (version 5.0) (Pang et al., 2021). The human

metabolome database (Wishart et al., 2018) and Metlin database (Guijas et al., 2018) were used to search unknown polar metabolite features based on their m/z with a mass error value of less than 15 ppm.

Similar procedures as plasma non-polar sample preparations, LC-MS run and data processing were followed for milk lipidomics analysis except for samples volume, i.e., 200 µL each of extracted samples was used for milk lipidomics analysis.

### 3.2.6 Statistical analysis

The concentration of amino acids in plasma samples and three batches of each milk samples and intake of amino acids and lipids in the last meal were analysed using one-way analysis of variance (ANOVA) from the rstatix package for R (version 4.02). Amino acid concentrations with an FDR $< 0.05$ between milk treatments were considered significantly different. The Fisher's least significant difference test was used for posthoc analysis, performed using the agricolae package for R.

Milk lipids' relative intensities data were expressed as relative percentage (%) for calculating the lipid intake in the last meal. The percentage of each lipid was calculated within each lipid class. Lipids within the same class, e.g., TGs, ionise at a similar rate, whereas lipids belonging to different classes ionise differently in the electrospray source; hence, the lipids were not expressed as a percentage of total lipids but as a percentage within the lipid class.

Multivariate statistical analyses, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were conducted to investigate differences in polar and non-polar metabolite profiles in plasma samples in response to different milk treatments using SIMCA (version 16). PCA was used to analyse the overall

variation in samples, whereas PLS-DA, a supervised clustering method, discriminated between samples based on class information. Validation of the PLS-DA model was performed using the predictive ability of the model ($Q^2$) and analysis of variance of cross-validated residuals (CV-ANOVA), i.e., $Q^2$ value approximately > 0.5 and CV-ANOVA P-value < 0.05, were considered as a good model for multivariate data. Permutation tests involving 100 permutations were used to check the robustness of the model. Features with a variable important for the projection (VIP) score >1 identified using the PLS-DA model were used for metabolite selection.

One-way ANOVA was conducted using the Metaboanalyst platform (version 5.0) (Pang et al., 2021). Metabolites with an FDR < 0.05 were considered significant. In addition, a pair-wise plasma metabolite relative intensity fold change (FC) was calculated between milk treatment groups (ovine vs caprine, ovine vs bovine, and caprine vs bovine). $Log_2FC$ > 1 (higher relative intensity) or $Log_2FC$ < -1 (lower relative intensity) (equivalent to FC > 2) and FDR < 0.05, identified using a t-test, was considered significant. Metaboanalyst (version 5.0) was used for heatmap visualisation, and Ward's Method clustering algorithm was used for hierarchical clustering analysis.

The association between milk nutrient intake (amino acids and lipids) in the last meal and plasma metabolites were assessed using Spearman correlations. Correlations with P < 0.05 and rho > 0.5 or rho < -0.5 were considered significant and visualised using the corrplot package in R. Last meal intake was used for correlation analysis as the nutrient appearance in the peripheral blood is expected to be due to the last meal after 18 h of fasting.

## 3.3 Results

### 3.3.1 Milk amino acid profiles

Ovine milk had significantly higher (FDR < 0.05) concentrations of individual amino acids, total amino acid (TAA), essential amino acid (EAA), branched-chain amino acid (BCAA), and large neutral amino acid (LNAA) compared to bovine milk and caprine milk (Error! Reference source not found.). Bovine milk had significantly higher (FDR < 0.05) concentrations of individual amino acids, TAA, EAA, BCAA, and LNAA than caprine milk, except cysteine, proline, threonine, and valine (FDR > 0.05).

For the last feed, protein contents (2 g of protein per kg BW) (Table 3-2) were matched between milk treatments, resulting in similar amino acid intakes between milk treatments (Table 3-1). Only threonine intake differed between milk treatments, i.e., piglets fed caprine milk consumed more threonine than bovine and ovine milk groups.

**Table 3-1 Amino acid composition of bovine, caprine, and ovine raw whole milk, and amino acid provided in the last meal***

| Amino acid** | Raw whole milk (mg/mL) | | | Intake (mg) | | |
|---|---|---|---|---|---|---|
| | Bovine | Caprine | Ovine | Bovine | Caprine | Ovine |
| Alanine | 1.10±0.02[b] | 0.90±0.08[c] | 2.13±0.06[a] | 288.96±40.35 | 289.5±33.24 | 336.52±37.13 |
| Arginine | 1.16±0.07[b] | 0.87±0.09[c] | 2.04±0.13[a] | 303.81±42.43 | 278.42±31.96 | 322.42±35.58 |
| Aspartic acid | 2.54±0.13[b] | 2.15±0.07[c] | 4.56±0.04[a] | 667.75±93.25 | 692.58±79.52 | 720.64±79.52 |
| Cysteine | 0.21±0.01[b] | 0.21±0.02[b] | 0.39±0.03[a] | 55.94±7.81 | 65.5±7.52 | 62.23±6.87 |
| Glutamic acid | 6.88±0.13[b] | 5.93±0.16[c] | 11.47±0.45[a] | 1805.17±252.09 | 1907.77±219.03 | 1813.03±200.05 |
| Glycine | 0.70±0.01[b] | 0.55±0.03[c] | 1.21±0.02[a] | 184.58±25.78 | 178.57±20.50 | 191.48±21.13 |
| Histidine | 0.85±0.03[b] | 0.75±0.03[c] | 1.48±0.04[a] | 224.28±31.32 | 241.86±27.77 | 233.88±25.81 |
| Isoleucine | 1.69±0.07[b] | 1.39±0.07[c] | 2.74±0.02[a] | 443.37±61.91 | 448.37±51.48 | 432.95±47.77 |
| Leucine | 3.20±0.15[b] | 2.75±0.05[c] | 5.53±0.22[a] | 839.08±117.18 | 885.55±101.67 | 874.36±96.48 |
| Lysine | 2.72±0.06[b] | 2.36±0.04[c] | 4.65±0.23[a] | 712.68±99.52 | 758.6±87.10 | 734.54±81.05 |
| Methionine | 0.89±0.03[b] | 0.74±0.02[c] | 1.60±0.06[a] | 232.32±32.44 | 236.76±27.18 | 252.43±27.85 |
| Phenylalanine | 1.57±0.07[b] | 1.40±0.02[c] | 2.61±0.06[a] | 410.83±57.37 | 450.45±51.72 | 412.3±45.50 |
| Proline | 3.31±0.09[b] | 3.11±0.14[b] | 5.91±0.18[a] | 869.33±121.40 | 999.49±114.75 | 933.48±103.00 |
| Serine | 1.74±0.06[b] | 1.43±0.05[c] | 2.82±0.12[a] | 456.41±63.74 | 459.76±52.79 | 445.45±49.15 |
| Threonine | 1.54±0.10[b] | 1.53±0.08[b] | 2.38±0.09[a] | 403.85±56.40[b] | 492.9±56.59[a] | 375.54±41.44[b] |

| | | | | | | |
|---|---|---|---|---|---|---|
| Tryptophan | 0.47±0.01[b] | 0.40±0.02[c] | 0.83±0.01[a] | 122.84±17.15 | 129.93±14.92 | 131.61±14.52 |
| Tyrosine | 1.68±0.06[b] | 1.16±0.06[c] | 2.67±0.09[a] | 440.46±61.51 | 371.69±42.67 | 421.63±46.52 |
| Valine | 2.17±0.09[b] | 2.09±0.08[b] | 3.69±0.12[a] | 568.68±79.42 | 671.03±77.04 | 583.54±64.39 |
| TAA | 34.41±1.02[b] | 29.71±0.85[c] | 58.71±1.64[a] | 9030.33±1261.06 | 9558.71±1097.45 | 9278.05±1023.73 |
| EAA | 15.08±0.57[b] | 13.41±0.29[c] | 25.51±0.75[a] | 3957.92±552.71 | 4315.44±495.46 | 4031.17±444.80 |
| BCAA | 7.05±0.31[b] | 6.23±0.20[c] | 11.96±0.35[a] | 1851.13±258.51 | 2004.94±230.19 | 1890.85±208.64 |
| LNAA | 14.04±0.57[b] | 12.21±0.26[c] | 23.53±0.60[a] | 3685.7±514.70 | 3928.52±451.04 | 3718.26±410.27 |

**Data analysed via one-way ANOVA with post hoc Fisher's least significant difference test. **Values are represented as mean ± standard deviation and means for composition (number of samples=3 per group) were compared separately to means for intake (number of samples=8 per group). [a-c] Values in the same row without a common superscript are different (FDR <0.05) from each other. TAA, total amino acid; EAA, essential amino acid; BCAA, branched-chain amino acid; LNAA, large neutral amino acids.*

**Table 3-2 Milk composition of bovine, caprine, and ovine raw whole milk and amounts provided in the last meal***

| Component** | Raw milk (g/100ml) | | | Intake (g) | | |
|---|---|---|---|---|---|---|
| | Bovine | Caprine | Ovine | Bovine | Caprine | Ovine |
| Dry matter | 13.10± 0.20[b] | 11.23 ± 0.31[c] | 17.6 0± 0.10[a] | 34.38 ± 4.80[a] | 36.15 ± 4.15[a] | 27.82 ± 3.07[b] |
| Protein | 3.61 ± 0.07[b] | 3.16 ± 0.11[c] | 6.27 ± 0.08[a] | 9.50 ± 1.33 | 10.20 ± 1.17 | 9.93 ± 1.10 |
| Fat | 4.06 ± 0.27[b] | 3.22 ± 0.17[c] | 6.31 ± 0.09[a] | 10.66 ± 1.49 | 10.39 ± 1.19 | 9.97 ± 1.10 |
| Lactose | 4.56 ± 0.09[a] | 3.91 ± 0.07[c] | 4.16 ± 0.06[b] | 11.97 ± 1.67[a] | 12.61 ± 1.45[a] | 6.59 ± 0.73[b] |
| Gross energy | 75.80 ± 1.84[b] | 61.11 ± 1.23[c] | 107.50 ± 0.27[a] | 198.95 ± 27.78 | 196.63 ± 22.58 | 169.90 ± 18.75 |

**Data analysed via one-way ANOVA with post hoc Fisher's least significant difference test. **Values are represented as mean ± standard deviation of three batches of each milk type and nutrient intake of piglets. [a-c] Values in the same row without a common superscript are different (FDR <0.05) from each other.*

### 3.3.2 Milk lipid profiles

Four hundred and ninety-five features were detected during the initial lipidomics data analysis. After filtration and removal of background noise, 88 features were identified and used for subsequent statistical analyses.

The PCA score plot showed a clear separation between milk (Figure 3-2A). A hierarchical cluster analysis confirmed the lipids grouping by the PCA model (Figure 3-2B). One-way ANOVA analysis showed that 81 out of 88 lipids significantly differed between milk (Appendix Table A2). These selected lipids were used further for pair-wise comparison between milk groups, as shown in Appendix Table A2. The relative intensity of 22 TG in ovine milk was significantly higher ($log_2FC > 1$ and $FDR < 0.05$) than in bovine milk (Appendix Table A2). The relative intensity of 36 TG in ovine milk was significantly higher than in caprine milk (Appendix Table A2). The relative intensity of the lipids between bovine and caprine milk was similar.

As per the study design, piglets received different volumes of each milk type to compensate for the balanced protein intake, resulting in almost similar total fat intake between the milk treatments (Table 3-2). However, there were differences in the intake of specific lipids between milk treatments (Table 3-3). For example, piglets fed ovine milk generally had a higher TG intake than caprine or bovine milk groups. Similarly, piglets fed with caprine milk generally had a lower intake of TG than those fed ovine or bovine milk.

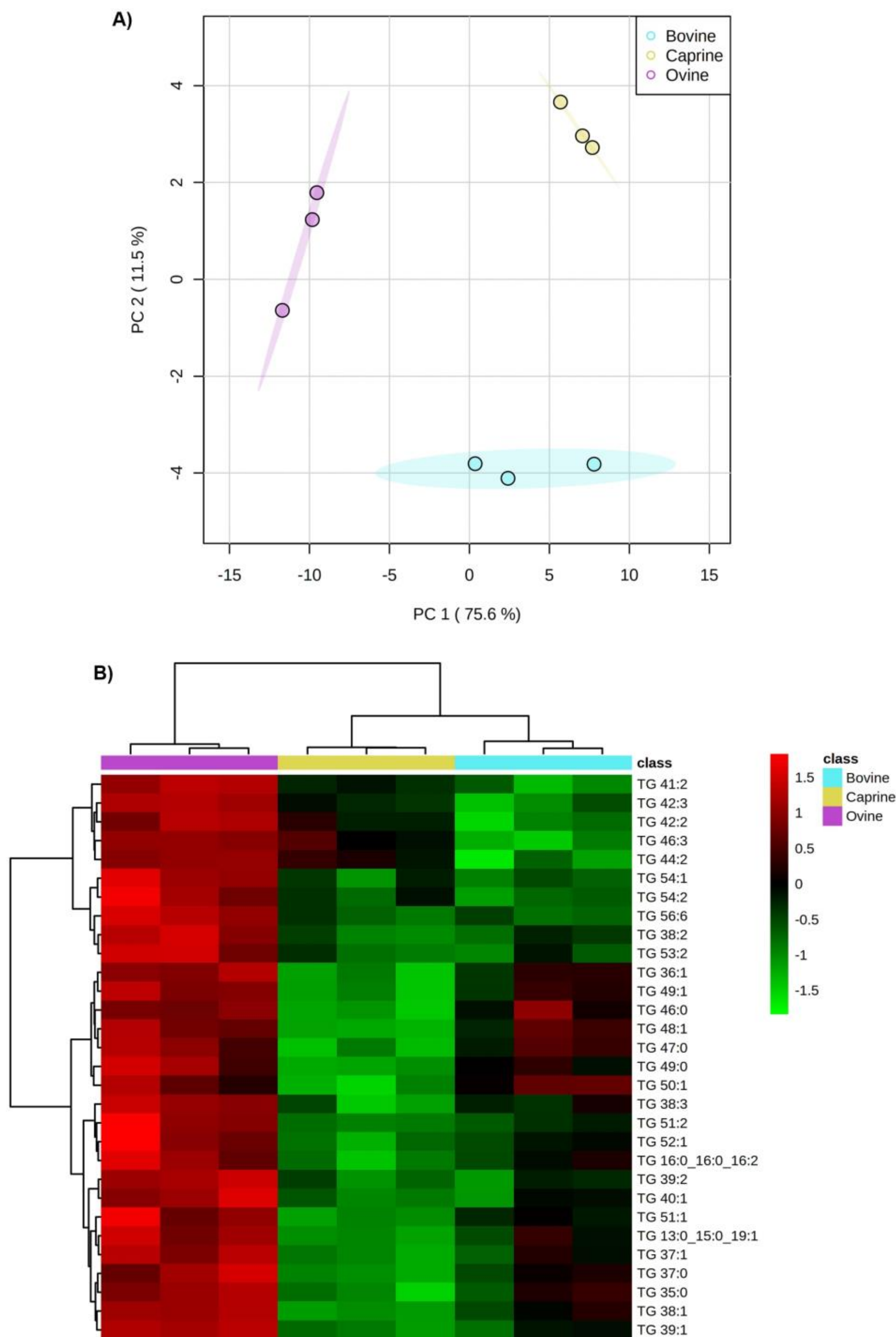



**Figure 3-2 Lipid relative intensity differences between bovine, caprine and ovine milk.**

A) Score plot of principal component analysis showing lipid relative intensity differences between bovine, caprine and ovine milk. The first two principal components (PC) are plotted. Percentages of variation explained by each principal component are indicated along the axes. B) Heatmap showing hierarchical clustering [method, Ward] of top 30 significantly different lipids between bovine (aqua), caprine (yellow), and ovine (purple) milk samples. Heatmap colour indicates normalised (Z score) peak intensity of lipids. The intensity of the red colour denotes the number of standard deviations above the mean (higher relative intensity), and the intensity of the green colour denotes the number of standard deviations below the mean (lower relative intensity). TG, triglyceride.

**Table 3-3 Lipid composition of bovine, caprine, and ovine raw whole milk and lipids provided in the last meal**

| Lipid* | Raw whole milk (%) | | | Intake** (mg)+ | | |
|---|---|---|---|---|---|---|
| | Bovine | Caprine | Ovine | Bovine | Caprine | Ovine |
| TG 8:0_12:0_14:0 | 3.00 | 2.51 | 3.05 | 319.2±44.62[a] | 261.24±29.99[b] | 303.63±33.48[a] |
| TG 9:0_9:0_16:0 | 1.66 | 1.39 | 1.67 | 176.97±24.74[a] | 144.96±16.64[b] | 166.48±18.36[a] |
| TG 10:0_14:0_16:0 | 2.34 | 2.86 | 2.52 | 248.83±34.78[b] | 297.35±34.13[a] | 251.75±27.76[b] |
| TG 12:0_12:0_16:0 | 1.26 | 1.47 | 1.40 | 134.37±18.78 | 152.41±17.49 | 139.54±15.39 |
| TG 12:0_14:0_16:0 | 2.18 | 2.74 | 2.31 | 232.66±32.52[b] | 284.92±32.70[a] | 230.19±25.38[b] |
| TG 13:0_15:0_19:1 | 0.62 | 0.51 | 0.65 | 65.93±9.22[a] | 52.90±6.07[b] | 65.23±7.17[a] |
| TG 14:0_14:0_14:0 | 0.90 | 1.21 | 0.96 | 95.43±13.34[b] | 125.39±14.39[a] | 95.26±10.5[b] |
| TG 14:0_16:0_18:2 | 1.99 | 1.71 | 1.71 | 211.55±29.57[a] | 177.96±20.43[b] | 170.28±18.78[b] |
| TG 14:0_18:1_18:1 | 4.32 | 3.94 | 3.63 | 460.28±64.34[a] | 409.15±46.96[ab] | 362.04±39.92[b] |
| TG 16:0_16:0_16:2 | 0.46 | 0.43 | 0.43 | 49.12±6.85 | 44.68±5.13 | 42.92±4.73 |
| TG 16:0_16:0_18:2 | 0.60 | 0.55 | 0.50 | 63.95±8.94[a] | 57.22±6.57[ab] | 50.18±5.53[b] |
| TG 16:0_16:0_20:3 | 0.36 | 0.42 | 0.41 | 38.63±5.40 | 43.36±4.97 | 40.86±4.50 |
| TG 16:0_18:1_18:2 | 2.17 | 2.59 | 2.73 | 230.74±32.26[b] | 268.71±30.84[a] | 271.8±29.97[a] |
| TG 26:0 | 0.06 | 0.07 | 0.15 | 6.10±0.85[b] | 7.07±0.81[b] | 15.16±1.67[a] |
| TG 28:0 | 0.16 | 0.15 | 0.33 | 32.93±2.37[a] | 15.85±1.82[b] | 32.43±3.58[a] |
| TG 30:0 | 0.21 | 0.31 | 0.38 | 38.63±3.74[a] | 32.63±3.74[b] | 37.70±4.16[a] |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 32:0 | 0.43 | 0.35 | 0.50 | 45.34±6.34$^{a}$ | 36.11±4.15$^{b}$ | 49.36±5.44$^{a}$ |
| TG 32:1 | 0.11 | 0.20 | 0.16 | 11.53±1.61$^{c}$ | 20.45±2.35$^{a}$ | 15.62±1.72$^{b}$ |
| TG 33:0 | 0.14 | 0.09 | 0.16 | 14.73±2.06$^{a}$ | 9.87±1.13$^{b}$ | 16.15±1.78$^{a}$ |
| TG 35:0 | 0.61 | 0.45 | 0.63 | 64.55±9.02$^{a}$ | 47.02±5.40$^{b}$ | 63.07±6.95$^{a}$ |
| TG 36:0 | 2.84 | 2.28 | 2.29 | 303.08±42.37$^{a}$ | 236.99±27.21$^{b}$ | 228.55±25.20$^{b}$ |
| TG 36:1 | 2.12 | 1.66 | 1.96 | 225.35±31.52$^{a}$ | 172.84±19.84$^{b}$ | 195.53±21.56$^{b}$ |
| TG 37:0 | 0.64 | 0.53 | 0.67 | 68.57±9.59$^{a}$ | 54.82±6.29$^{b}$ | 66.91±7.38$^{a}$ |
| TG 37:1 | 0.36 | 0.29 | 0.45 | 38.75±5.42$^{b}$ | 30.21±3.47$^{c}$ | 44.84±4.95$^{a}$ |
| TG 38:0 | 3.59 | 3.30 | 3.27 | 382.12±53.42 | 343.03±39.37 | 326.22±35.97 |
| TG 38:1 | 2.05 | 1.82 | 1.96 | 218.02±30.48 | 188.68±21.66 | 195.39±21.55 |
| TG 38:2 | 0.89 | 0.92 | 1.21 | 94.36±13.19$^{b}$ | 95.85±11$^{b}$ | 120.48±13.28$^{a}$ |
| TG 38:3 | 0.11 | 0.06 | 0.22 | 11.61±1.62$^{b}$ | 6.62±0.76$^{c}$ | 21.53±2.37$^{a}$ |
| TG 39:0 | 0.37 | 0.39 | 0.49 | 39.94±5.58$^{b}$ | 40.18±4.61$^{b}$ | 48.68±5.37$^{a}$ |
| TG 39:1 | 0.38 | 0.36 | 0.48 | 40.78±5.7$^{b}$ | 37.33±4.28$^{b}$ | 47.96±5.29$^{a}$ |
| TG 39:2 | 0.08 | 0.08 | 0.19 | 8.74±1.22$^{b}$ | 8.50±0.97$^{b}$ | 18.85±2.08$^{a}$ |
| TG 40:1 | 2.78 | 2.87 | 3.02 | 296.12±41.38 | 298.65±34.28 | 301.46±33.24 |
| TG 41:0 | 0.32 | 0.38 | 0.43 | 34.51±4.82$^{b}$ | 39.85±4.57$^{a}$ | 42.54±4.69$^{a}$ |
| TG 41:1 | 0.21 | 0.28 | 0.29 | 22.68±3.17$^{b}$ | 29.59±3.41$^{a}$ | 29±3.20$^{a}$ |
| TG 41:2 | 0.03 | 0.07 | 0.14 | 3.69±0.52$^{c}$ | 7.53±0.86$^{b}$ | 13.63±1.52$^{a}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 42:1 | 1.65 | 2.35 | 2.04 | 175.85±24.58$^{c}$ | 244.39±28.05$^{a}$ | 203.78±22.47$^{b}$ |
| TG 42:2 | 0.71 | 1.14 | 0.92 | 75.15±10.50$^{c}$ | 118.23±13.57$^{a}$ | 91.63±10.10$^{b}$ |
| TG 42:3 | 0.23 | 0.37 | 0.39 | 24.49±3.42$^{b}$ | 38.34±4.40$^{a}$ | 39.2±4.32$^{a}$ |
| TG 43:0 | 0.36 | 0.39 | 0.43 | 38.74±5.41 | 40.93±4.71 | 43.07±4.75 |
| TG 43:1 | 0.23 | 0.26 | 0.39 | 24.14±3.37$^{b}$ | 27.01±3.11$^{b}$ | 38.67±4.26$^{a}$ |
| TG 43:2 | 0.05 | 0.05 | 0.10 | 4.93±0.69$^{b}$ | 5.02±0.58$^{b}$ | 9.55±1.05$^{a}$ |
| TG 44:0 | 2.53 | 2.66 | 2.22 | 269.34±37.65$^{a}$ | 276.79±31.77$^{a}$ | 221.71±24.45$^{b}$ |
| TG 44:1 | 2.01 | 3.10 | 2.49 | 213.73±29.88$^{c}$ | 321.8±36.94$^{a}$ | 247.8±27.32$^{b}$ |
| TG 44:2 | 0.62 | 1.21 | 0.96 | 65.97±9.22$^{c}$ | 125.51±14.41$^{a}$ | 95.71±10.55$^{b}$ |
| TG 44:3 | 0.20 | 0.34 | 0.34 | 21.46±3.01$^{b}$ | 35.41±4.07$^{a}$ | 33.44±3.69$^{a}$ |
| TG 45:0 | 0.60 | 0.45 | 0.53 | 63.40±8.86$^{a}$ | 46.48±5.34$^{b}$ | 52.47±5.79$^{b}$ |
| TG 45:1 | 0.32 | 0.33 | 0.37 | 33.99±4.75 | 34.61±3.97 | 36.93±4.07 |
| TG 46:0 | 3.21 | 2.55 | 2.42 | 341.84±47.79$^{a}$ | 265.09±30.43$^{b}$ | 241.07±26.58$^{b}$ |
| TG 46:1 | 3.24 | 3.20 | 2.81 | 345.39±48.28$^{a}$ | 332.3±38.14$^{a}$ | 279.74±30.85$^{b}$ |
| TG 46:2 | 0.94 | 1.50 | 1.16 | 100.07±13.99$^{b}$ | 155.45±17.85$^{a}$ | 115.23±12.71$^{b}$ |
| TG 46:3 | 0.24 | 0.51 | 0.41 | 25.13±3.51$^{c}$ | 52.63±6.04$^{a}$ | 40.98±4.52$^{b}$ |
| TG 47:0 | 0.78 | 0.55 | 0.65 | 82.94±11.59$^{a}$ | 57.04±6.55$^{b}$ | 65.22±7.19$^{b}$ |
| TG 48:0 | 2.75 | 2.17 | 2.00 | 13.15±1.20$^{a}$ | 10.66±1.22$^{b}$ | 13.05±1.44$^{a}$ |
| TG 48:1 | 6.11 | 4.71 | 4.89 | 293.15±40.98$^{a}$ | 225.13±25.84$^{b}$ | 199.57±22.01$^{b}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 48:3 | 0.41 | 0.37 | 0.40 | 43.66±6.11 | 38.19±4.38 | 40.04±4.41 |
| TG 49:0 | 0.58 | 0.47 | 0.52 | 61.55±8.61$^{a}$ | 48.49±5.57$^{b}$ | 52.22±5.76$^{b}$ |
| TG 49:1 | 1.32 | 0.95 | 1.28 | 140.15±19.59$^{a}$ | 98.88±11.35$^{b}$ | 127.37±14.05$^{a}$ |
| TG 49:2 | 0.43 | 0.36 | 0.45 | 45.88±6.41$^{a}$ | 37.77±4.34$^{b}$ | 44.4±4.90$^{a}$ |
| TG 49:3 | 0.06 | 0.07 | 0.13 | 6.32±0.89$^{c}$ | 7.73±0.89$^{b}$ | 12.91±1.42$^{a}$ |
| TG 50:0 | 1.92 | 1.80 | 1.39 | 204.31±28.56$^{a}$ | 186.66±21.43$^{a}$ | 138.8±15.31$^{b}$ |
| TG 50:1 | 7.69 | 6.01 | 5.48 | 819.04±114.49$^{a}$ | 624.69±71.71$^{b}$ | 546.23±60.23$^{b}$ |
| TG 50:3 | 1.06 | 0.96 | 1.17 | 113.23±15.83$^{ab}$ | 99.39±11.41$^{b}$ | 117.14±12.92$^{a}$ |
| TG 50:4 | 0.20 | 0.18 | 0.25 | 21.29±2.98$^{b}$ | 18.93±2.17$^{b}$ | 24.97±2.75$^{a}$ |
| TG 51:0 | 0.29 | 0.27 | 0.31 | 30.58±4.27 | 27.62±3.17 | 31.33±3.46 |
| TG 51:1 | 0.99 | 0.89 | 1.01 | 105.82±14.79 | 92.03±10.57 | 101.19±11.16 |
| TG 51:2 | 0.74 | 0.73 | 0.94 | 78.72±11.01$^{b}$ | 75.49±8.66$^{b}$ | 93.93±10.36$^{a}$ |
| TG 51:3 | 0.20 | 0.22 | 0.31 | 21.46±3.01$^{b}$ | 23.29±2.67$^{b}$ | 30.94±3.41$^{a}$ |
| TG 52:1 | 3.57 | 3.65 | 3.23 | 380.39±53.17$^{a}$ | 378.97±43.5$^{a}$ | 322.37±35.55$^{b}$ |
| TG 52:2 | 5.63 | 5.79 | 5.60 | 599.86±83.86 | 602.27±69.13 | 557.89±61.52 |
| TG 52:4 | 0.67 | 0.68 | 0.99 | 71.02±9.93$^{b}$ | 70.51±8.1$^{b}$ | 98.43±10.86$^{a}$ |
| TG 52:5 | 0.11 | 0.07 | 0.20 | 11.35±1.59$^{b}$ | 7.11±0.81$^{c}$ | 19.85±2.19$^{a}$ |
| TG 53:2 | 0.41 | 0.47 | 0.48 | 43.30±6.05 | 48.46±5.56 | 47.48±5.24 |
| TG 53:3 | 0.18 | 0.21 | 0.29 | 19.44±2.72$^{b}$ | 21.99±2.53$^{b}$ | 28.69±3.16$^{a}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 53:4 | 0.04 | 0.06 | 0.10 | 4.41±0.62[c] | 6.24±0.72[b] | 9.77±1.08[a] |
| TG 54:0 | 0.12 | 0.14 | 0.14 | 12.76±1.78 | 14.38±1.65 | 14.06±1.55 |
| TG 54:1 | 0.64 | 0.80 | 0.81 | 68.13±9.52[b] | 83.16±9.54[a] | 81.17±8.95[a] |
| TG 54:2 | 1.47 | 2.02 | 2.00 | 156.79±21.92[b] | 209.86±24.09[a] | 199.73±22.02[a] |
| TG 54:3 | 1.69 | 2.51 | 2.31 | 179.91±25.15[c] | 261.31±29.99[a] | 230.14±25.38[b] |
| TG 54:4 | 0.81 | 1.24 | 1.27 | 86.23±12.05[b] | 129.32±14.84[a] | 126.53±13.95[a] |
| TG 54:5 | 0.33 | 0.46 | 0.52 | 34.87±4.88[b] | 48.31±5.54[a] | 51.99±5.73[a] |
| TG 56:6 | 0.03 | 0.04 | 0.14 | 3.45±0.48[b] | 4.08±0.47[b] | 13.56±1.50[a] |

**Only the lipids whose relative intensities were significant between milk are represented as a percentage. ** Data analysed via one-way ANOVA with post hoc Fisher's least significant difference test and values are represented as mean ± standard deviation. [a-c] Values in the same row without a common superscript are different (FDR <0.05) from each other. [+]Intake is represented as the amount of each TG in the total amount of fat consumed in the last meal. TG, Triglycerides; DG, diglycerides*

### 3.3.3 Plasma polar metabolites

The number of plasma features obtained in positive and negative ionisation modes from the polar analytical stream was 621 and 120, respectively. The positive and negative ionisation data were combined into one data set after filtration and background noise removal. The resulting 318 plasma features (43 known and 275 unknown) were used for multivariate analyses. The PCA model could not resolve any separation of polar plasma metabolites between milk treatments (Appendix Figure A1A). However, a validated PLS-DA model ($Q2 = 0.81$ and CV-ANOVA P-value $< 0.05$) with a good fit showed a clear and robust separation of polar plasma metabolites between milk treatments (Figure 3-3A-B).

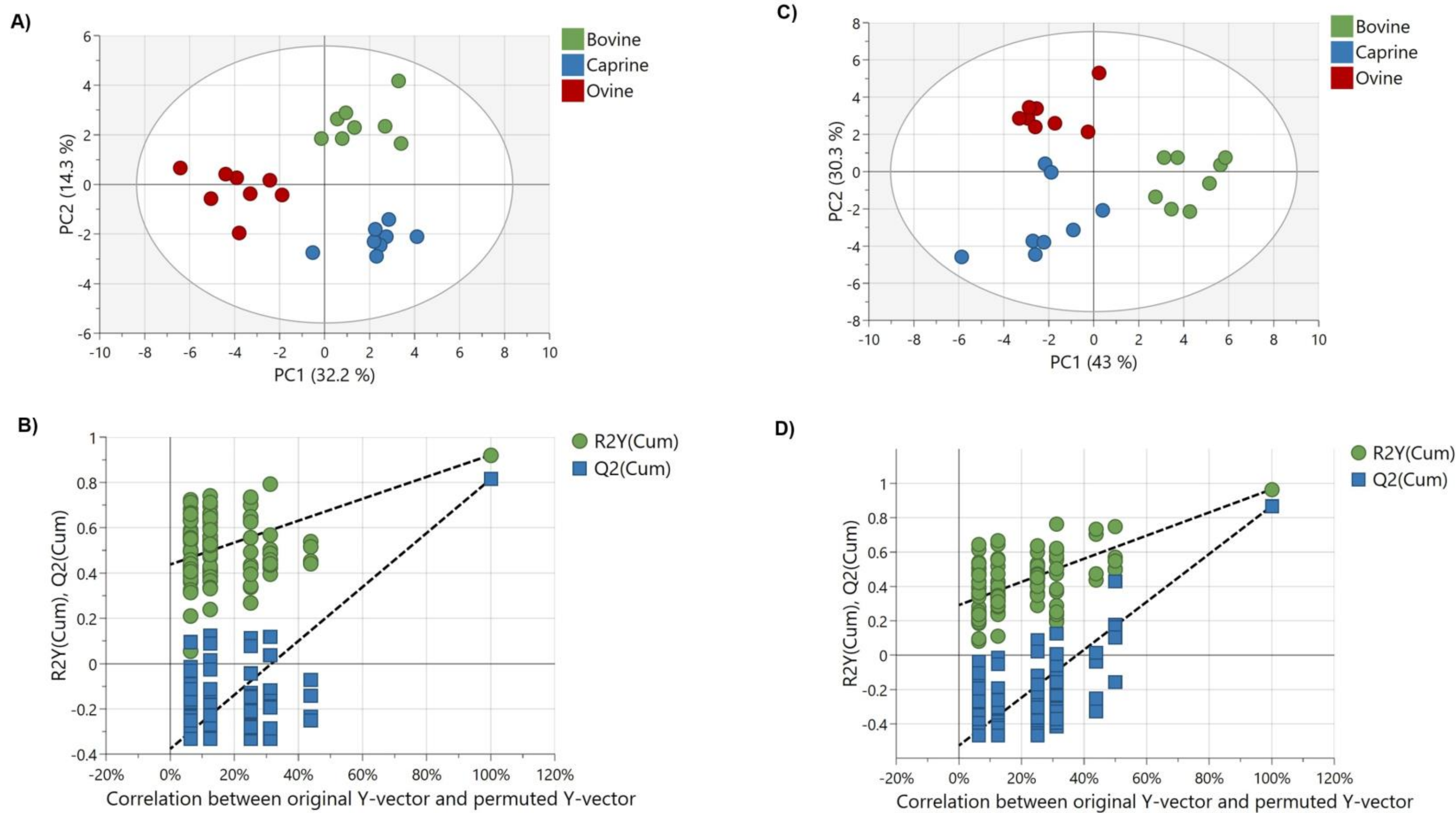



**Figure 3-3 Partial least squares discriminant analysis (PLS-DA) of metabolites relative intensity differences in plasma of piglets fed bovine, caprine, or ovine milk treatment.**

A) Score plot and B) permutation plot of polar metabolites. C) Score plot and D) permutation plot of non-polar metabolites. Permutation plots involve 100 permutation tests showing no overfitting of the PLS-DA model, confirming the robustness of the model. The criteria for evaluating whether there is overfitting of the PLS-DA model is that Q2 and R2 values of the permutated Y model to the left were lower than the Q2 and R2 value of the original model to the far right. The first two principal components (PC) are plotted. Percentages of variation explained by each PC are indicated along the axes. PC, principal component.

The relative intensity of seven polar plasma metabolites was significantly different (FDR < 0.05) between milk treatments and contributed the most to the separation between milk treatments (VIP > 1) (Table 3-4). Out of the seven features, three features were unknown during the initial processing, which were putatively identified using HMDB search as phenyl pyruvic acid (measured mass (m/z): 165.0533, retention time (min): 8.81), LPC (18:1) (actual mass: 544.3366, retention time: 5.619), and lysophosphatidylethanolamine (LPE) (18:0) (actual mass: 482.3202, retention time: 5.798). The relative intensity of acetylcarnitine and LPC (18:1) in the ovine milk group was significantly higher than in the bovine milk group (Table 3-4). No metabolite FC were significant between ovine and caprine milk groups and between caprine and bovine milk groups (Table 3-4).

Overall, the relative intensity of plasma polar metabolites in the ovine milk group was higher compared to the caprine or bovine milk group (Figure 3-4A).

**Table 3-4 Polar metabolites with a significant difference in relative intensities in plasma of piglets fed bovine, caprine, or ovine milk treatment[+]**

| Polar metabolites | VIP | FDR | $Log_2$fold change | | |
|---|---|---|---|---|---|
| | | | Ovine vs Bovine | Ovine vs Caprine | Caprine vs Bovine |
| Acetylcarnitine | 2.85 | <0.01 | 1.05* | 0.81 | 0.24 |
| LysoPC (18:1) | 2.64 | <0.01 | 1.14* | 0.24 | 0.89 |
| Isoleucine | 2.52 | <0.01 | 0.36 | 0.50 | -0.14 |
| L-Tyrosine | 2.50 | 0.01 | 0.35 | 0.70 | -0.35 |
| Phenyl pyruvic acid | 2.40 | 0.01 | 0.41 | 0.71 | -0.30 |
| LysoPE (18:0) | 2.32 | 0.03 | 0.26 | 0.59 | -0.32 |
| Proline | 2.18 | 0.05 | 0.40 | 0.30 | 0.10 |

*[+]VIP value >1 from the partial least squares discriminant analysis model was used to select the major contributing polar metabolites. One-way ANOVA was used to determine the significant polar metabolites between milk treatments (FDR < 0.05). Only polar metabolites that satisfied multivariate and ANOVA analysis criteria are shown. *Polar metabolites with significant fold change > ± 1 (FDR < 0.05 using t-test) between treatments. FDR, false discovery rate; VIP, variable importance in projection; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine*

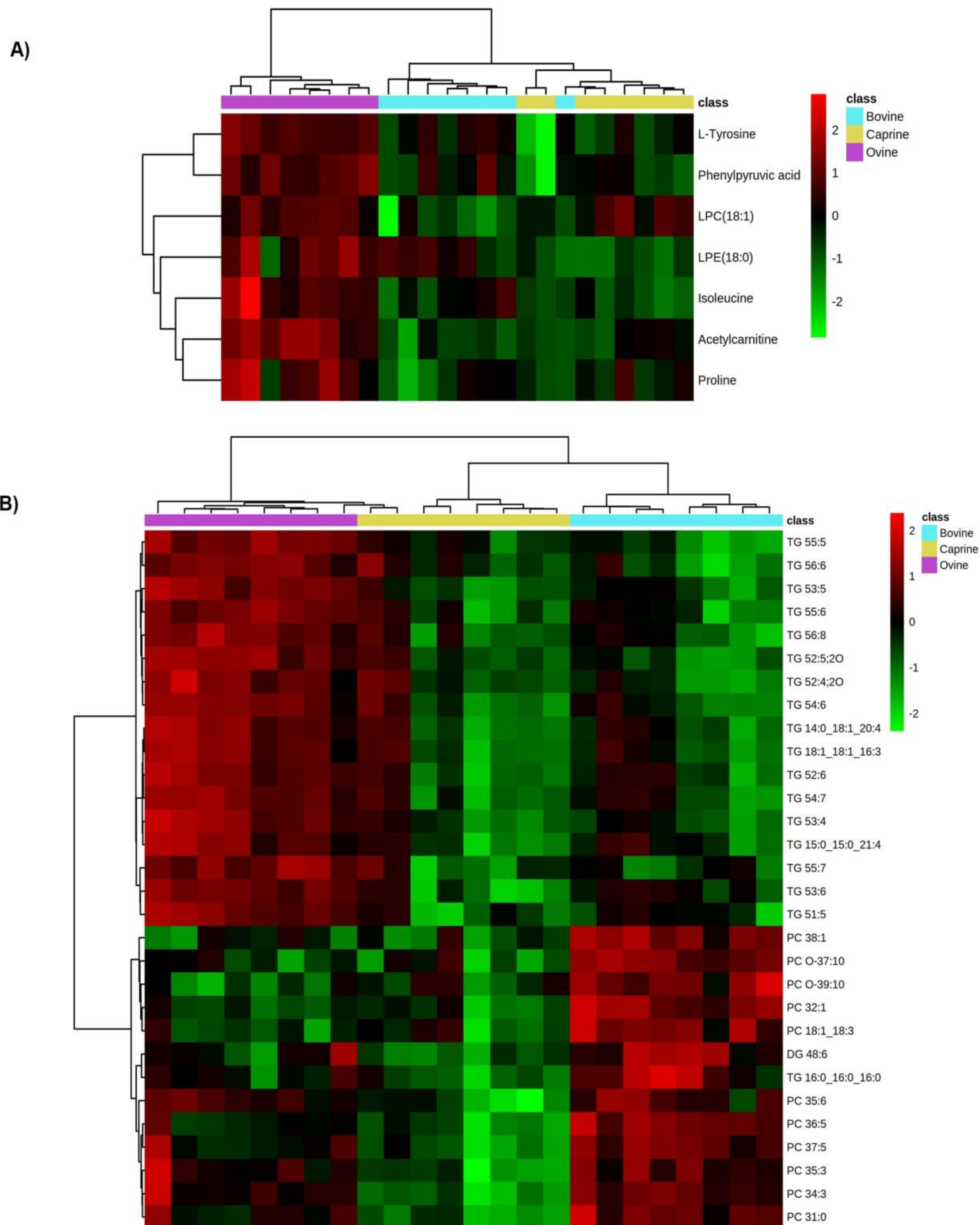



**Figure 3-4 Heatmap showing relative intensities of significantly different A) polar metabolites and B) lipids (top 30 significant lipids) in plasma of piglets fed bovine, caprine, or ovine milk treatment.**

Heatmap colour indicates normalised (Z score) peak intensity of metabolites. The intensity of the red colour denotes the number of standard deviations above the mean (higher relative intensity), and the intensity of the green colour denotes the number of standard deviations below the mean (lower relative intensity).

Most plasma polar metabolites identified using LC-MS-metabolomics were amino acids. Their concentrations were then quantified using the HPLC method. The plasma concentration of seven amino acids, namely asparagine, leucine, lysine, proline, taurine, threonine, and tyrosine, were significantly higher (FDR $< 0.05$) in the ovine milk treatment group compared to bovine or caprine milk treatment groups. (Table 3-5). In addition, the plasma concentration of taurine and threonine was higher in the caprine milk treatment group compared to the bovine or ovine milk treatment groups (Table 3-5).

**Table 3-5 Amino acid concentrations (μmol/L) in plasma of piglets fed bovine, caprine, or ovine milk treatment ***

| Amino acids** | Bovine | Caprine | Ovine | FDR |
|---|---|---|---|---|
| Alanine | 522.7±126.84 | 609.2±128.82 | 554.6±87.89 | 0.47 |
| Arginine | 59.59±5.12 | 56.89±10.19 | 81.09±29.46 | 0.08 |
| Asparagine | 68.93±17.11[b] | 70.54±7.62[b] | 94.03±13.22[a] | 0.01 |
| Aspartic acid | 11.64±3.17 | 9.43±2.59 | 13.44±4.18 | 0.18 |
| Citrulline | 124.9±37.21 | 118.6±19.73 | 122.9±23.64 | 0.98 |
| Cystine | 6.66±5.92 | 11.04±6.53 | 12.34±10.37 | 0.47 |
| Glutamic acid | 156.4±40.49 | 158.1±49.65 | 160.8±32.36 | 0.98 |
| Glutamine | 435.4±105.02 | 485.9±108.00 | 539.5±117.12 | 0.29 |
| Glycine | 1214.1±316.78 | 1385.8±223.85 | 1077.4±260.22 | 0.19 |
| Histidine | 48.83±14.23 | 46.11±8.61 | 49.95±7.91 | 0.93 |
| Hydroxyproline | 148.6±33.67 | 183.6±25.42 | 184.3±38.98 | 0.17 |
| Isoleucine | 147.3±42.75 | 119.1±11.49 | 145.8±21.75 | 0.2 |
| Leucine | 168.1±42.43[b] | 149.2±17.18[b] | 220.1±38.67[a] | 0.01 |
| Lysine | 151.6±51.86[b] | 145.4±39.91[b] | 226.8±58.53[a] | 0.03 |
| Methionine | 88.71±17.37 | 86.76±16.96 | 88.33±14.36 | 0.98 |
| 3-Methylhistidine | 15.76±9.86 | 9.66±5.84 | 15.48±4.37 | 0.29 |
| Ornithine | 69.51±21.47 | 51.64±10.33 | 80.41±20.64 | 0.05 |

| | | | | |
|---|---|---|---|---|
| Phenylalanine | 62.55±14.52 | 62.28±11.03 | 72.33±9.43 | 0.29 |
| Proline | 291.7±77.75[b] | 338.5±15.55[ab] | 385.8±51.81[a] | 0.03 |
| Serine | 164.1±51.81 | 174.8±26.26 | 168.3±14.95 | 0.95 |
| Taurine | 17.53±5.34[c] | 56.98±11.52[a] | 26.06±6.24[b] | 0.00 |
| Threonine | 153.5±31.72[b] | 419.7±118.92[a] | 205.45±71.29[b] | 0.00 |
| Tryptophan | 9.79±2.69 | 9.93±2.29 | 10.71±2.67 | 0.93 |
| Tyrosine | 124.4±30.24[a] | 92.88±24.69[b] | 148.2±14.89[a] | 0.01 |
| Valine | 288.1±62.5 | 339.2±49.32 | 362.8±57.39 | 0.11 |
| TAA | 4550.5±988.44 | 5191.2±401.62 | 5047.2±422.35 | 0.25 |
| EAA | 1118.4±226.15 | 1377.5±159.53 | 1382.5±209.90 | 0.07 |
| BCAA | 603.4±145.87 | 607.4±74.07 | 728.8±110.52 | 0.14 |
| LNAA | 1091.2±203.22 | 1324.9±152.79 | 1303.9±165.18 | 0.08 |

**Data analysed via one-way ANOVA with post hoc Fisher's least significant difference test. **Values are represented as mean ± standard deviation. [a-c] Values without a common superscript are different (FDR <0.05) from each other. FDR, false discovery rate. TAA, total amino acid; EAA, essential amino acid; BCAA, branched-chain amino acid; LNAA, large neutral amino acid*

### 3.3.4 Plasma non-polar metabolites

A total of 438 plasma features was obtained during the initial lipidomics data analysis of the non-polar (lipid) extracts. After filtration and removal of background noise, 324 plasma features were identified and used for subsequent statistical analyses.

The PCA model separated plasma lipid metabolite profiles between milk treatments. However, some overlap between milk treatments was observed (Appendix Figure A1B). Validated PLS-DA model ($Q2 = 0.86$ and CV-ANOVA P-value = $<0.001$) with a good fit showed clear and robust separation of plasma lipids between milk treatments (Figure 3-3C-D).

The relative intensity of 82 lipids was significantly different (FDR $< 0.05$) between milk treatments and contributed the most to the separation between milk treatments (VIP $> 1$) (Table 3-6). Therefore, these selected lipids were used further for pair-wise comparison between milk groups (Table 3-6).

The relative intensity of 11 lipids (TG) in the ovine milk group was significantly higher than in the bovine milk group (Table 3-6). In contrast, the relative intensity of two phospholipids (PC 38:1, PC O-36:1) in the ovine milk group was significantly lower ($\log_2$FC $< -1$ & FDR $< 0.05$) than in the bovine milk group (Table 3-6). The relative intensity of 13 lipids (1 phospholipid (PC 39:6) and 12 TG) in the ovine milk group was significantly higher than in the caprine milk group (Table 3-6). Conversely, the relative intensity of 12 lipids (6 phospholipids, 1 sphingolipid (SM 41:2;2O), 5 TG) in the caprine milk group was significantly lower than in the bovine milk group (Table 3-6). Overall, the relative intensity pattern of plasma unsaturated TG obtained with the ovine milk treatment was distinct from caprine and bovine milk treatments, whereas the intensity

pattern of PC obtained with the bovine milk treatment was distinct from those from caprine and ovine milk treatments (Figure 3-4B).

**Table 3-6 Lipids with a significant difference in relative intensities in plasma of piglets fed bovine, caprine, or ovine milk treatment** +

| Lipids | VIP | FDR | Log2fold change | | |
|---|---|---|---|---|---|
| | | | Ovine vs Bovine | Ovine vs Caprine | Caprine vs Bovine |
| DG 48:6 | 1.23 | 0.03 | -0.44 | 0.19 | -0.62 |
| DG 48:9 | 1.1 | 0.05 | 0.98 | 0.59 | 0.39 |
| DG 49:6 | 1.42 | <0.01 | -0.68 | 0.27 | -0.95 |
| LPC 18:1/0:0 | 1.16 | 0.05 | -0.45 | 0.08 | -0.53 |
| LPC 20:4/0:0 | 1.13 | 0.05 | 0.78 | 0.26 | 0.52 |
| PC 30:0 | 1.34 | <0.01 | -0.50 | 0.34 | -0.84 |
| PC 31:0 | 1.67 | <0.01 | -0.33 | 0.66 | -0.99 |
| PC 31:2 | 1.09 | 0.04 | -0.7 | 0.61 | -1.31* |
| PC 32:0 | 1.15 | 0.02 | -0.36 | 0.4 | -0.75 |
| PC 32:1 | 1.64 | <0.01 | -0.73 | 0.07 | -0.79 |
| PC 33:1 | 1.28 | 0.01 | -0.47 | 0.43 | -0.9 |
| PC 33:3 | 1.27 | 0.01 | -1.14 | 1.04 | -2.18* |
| PC 34:1 | 1.37 | 0.01 | -0.38 | 0.07 | -0.45 |
| PC 34:3 | 1.69 | <0.01 | -0.14 | 0.97 | -1.11* |
| PC 35:0 | 1.13 | 0.03 | -0.43 | 0.34 | -0.77 |
| PC 35:3 | 1.38 | <0.01 | -0.08 | 0.62 | -0.7 |

| | | | | | |
|---|---|---|---|---|---|
| PC 35:4 | 1.31 | <0.01 | -0.14 | 0.51 | -0.65 |
| PC 35:5 | 1.27 | 0.01 | -0.77 | 0.74 | -1.52* |
| PC 35:6 | 1.34 | <0.01 | -0.06 | 0.72 | -0.78 |
| PC 36:1 | 1.51 | 0.04 | -0.84 | -0.1 | -0.74 |
| PC 16:0_20:4 | 1.11 | 0.04 | 0.45 | 0.23 | 0.21 |
| PC 18:1_18:3 | 1.51 | <0.01 | -0.41 | -0.01 | -0.4 |
| PC 36:5 | 1.65 | <0.01 | -0.89 | 0.67 | -1.55* |
| PC 37:4 | 1.08 | 0.03 | 0.87 | 0.6 | 0.27 |
| PC 37:5 | 1.51 | <0.01 | -0.33 | 0.56 | -0.9 |
| PC 38:1 | 1.55 | <0.01 | -1.71* | 0.13 | -1.85* |
| PC 38:2 | 1.06 | 0.03 | -0.27 | 0.33 | -0.6 |
| PC 38:4 | 1.28 | 0.04 | 0.97 | 0.28 | 0.7 |
| PC 39:6 | 1.01 | 0.01 | 0.82 | 1.07* | -0.25 |
| PC O-32:1 | 1.39 | 0.02 | -0.75 | 0.01 | -0.76 |
| PC O-36:1 | 1.24 | 0.04 | -1.05* | -0.27 | -0.78 |
| PC O-37:10 | 1.49 | <0.01 | -0.57 | 0.11 | -0.68 |
| PC O-39:10 | 1.47 | <0.01 | -0.22 | -0.05 | -0.17 |
| PC O-39:9 | 1.17 | 0.03 | -0.16 | 0.04 | -0.2 |
| PC O-41:10 | 1.45 | 0.03 | -0.32 | -0.03 | -0.29 |

| | | | | | |
|---|---|---|---|---|---|
| PE 36:1 | 1.25 | 0.04 | -1.6 | -0.03 | -1.57 |
| SM 32:1;2O | 1.43 | 0.01 | -0.84 | -0.08 | -0.76 |
| SM 33:1;2O | 1.2 | 0.02 | -0.69 | 0.29 | -0.98 |
| SM 36:1;2O | 1.46 | 0.01 | -0.83 | -0.07 | -0.76 |
| SM 38:7;3O | 1.35 | 0.01 | -0.57 | 0.11 | -0.69 |
| SM 39:7;2O | 1.41 | 0.02 | 0.8 | 0.36 | 0.44 |
| SM 40:2;2O | 1.21 | 0.05 | -0.48 | 0.18 | -0.66 |
| SM 41:2;2O | 1.08 | 0.04 | -0.62 | 1.02 | -1.64* |
| TG 14:0_15:0_18:1 | 1.00 | 0.01 | 0.04 | 0.94 | -0.9 |
| TG 14:0_16:0_18:0 | 1.27 | 0.01 | -0.64 | 0.55 | -1.19* |
| TG 14:0_18:1_20:4 | 1.07 | <0.01 | 1.08* | 1.16* | -0.08 |
| TG 15:0_15:0_21:4 | 1.02 | <0.01 | 0.8 | 1.06* | -0.26 |
| TG 15:0_17:0_17:0 | 1.14 | 0.01 | -0.35 | 0.52 | -0.87 |
| TG 16:0_16:0_15:1 | 1.1 | 0.01 | -0.17 | 0.6 | -0.77 |
| TG 16:0_16:0_16:0 | 1.37 | <0.01 | -0.52 | 0.37 | -0.89 |
| TG 16:0_16:0_17:0 | 1.04 | 0.03 | -0.32 | 0.67 | -0.99 |
| TG 17:0_17:0_17:3 | 1.21 | 0.01 | 1.05 | 1.53* | -0.48 |
| TG 18:1_18:1_16:3 | 1.05 | <0.01 | 0.83 | 0.85 | -0.02 |
| TG 45:0 | 1.11 | 0.01 | -0.26 | 0.93 | -1.18* |

| | | | | | |
|---|---|---|---|---|---|
| TG 46:0 | 1.24 | 0.01 | -0.59 | 0.59 | -1.18* |
| TG 47:0 | 1.23 | <0.01 | -0.38 | 0.85 | -1.23* |
| TG 48:4;1O | 1.18 | 0.01 | -0.38 | 0.73 | -1.11* |
| TG 49:1 | 1.01 | 0.01 | -0.02 | 0.8 | -0.82 |
| TG 49:3 | 1.02 | <0.01 | 0.68 | 1.24* | -0.56 |
| TG 49:4 | 1.06 | <0.01 | 1.22* | 1.86* | -0.63 |
| TG 50:1 | 1.1 | 0.03 | -0.18 | 0.33 | -0.51 |
| TG 51:5 | 1.32 | <0.01 | 1.50* | 1.81* | -0.31 |
| TG 52:4 | 1.00 | 0.01 | 0.71 | 0.66 | 0.04 |
| TG 52:6 | 1.06 | <0.01 | 1.03* | 1.24* | -0.21 |
| TG 53:4 | 1.26 | <0.01 | 0.84 | 0.83 | 0.02 |
| TG 53:5 | 1.37 | <0.01 | 1.05* | 1.11* | -0.06 |
| TG 53:6 | 1.34 | <0.01 | 1.17* | 1.83* | -0.66 |
| TG 54:4 | 1.06 | 0.04 | 0.59 | 0.37 | 0.22 |
| TG 54:5 | 1.13 | <0.01 | 0.88 | 0.67 | 0.21 |
| TG 54:6 | 1.13 | <0.01 | 0.85 | 0.83 | 0.02 |
| TG 54:7 | 1.05 | <0.01 | 1.02* | 1.12* | -0.1 |
| TG 55:5 | 1.48 | <0.01 | 1.07* | 0.73 | 0.35 |
| TG 55:6 | 1.14 | <0.01 | 0.97 | 0.93 | 0.04 |

| | | | | | |
|---|---|---|---|---|---|
| TG 55:7 | 1.47 | <0.01 | 1.14* | 1.12* | 0.03 |
| TG 56:5 | 1.29 | 0.01 | 0.6 | 0.23 | 0.37 |
| TG 56:6 | 1.33 | <0.01 | 0.85 | 0.5 | 0.35 |
| TG 56:7 | 1.08 | 0.01 | 0.71 | 0.59 | 0.12 |
| TG 56:8 | 1.13 | <0.01 | 1.17* | 1.04* | 0.12 |
| TG 58:6 | 1.14 | 0.01 | 0.84 | 0.82 | 0.02 |
| TG 58:7 | 1.02 | 0.02 | 0.39 | 0.28 | 0.11 |
| TG 58:8 | 1.23 | 0.01 | 0.85 | 0.43 | 0.42 |
| TG 58:9 | 1.17 | <0.01 | 1.29* | 0.92 | 0.37 |

*+VIP value >1 from the partial least squares discriminant analysis model was used to select the major contributing non-polar (lipids) metabolites. One-way ANOVA was used to determine the significant lipids between milk treatments (FDR < 0.05). Only lipids that satisfied multivariate and ANOVA analysis criteria are shown. *Lipids with significant $log_2$fold change > 1 or $log_2$fold change < -1 (FDR < 0.05 using t-test) between treatments. FDR, false discovery rate; VIP, variable importance in projection; DG, diglycerides; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PC-O, ether-linked phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TG, triglycerides.*

### 3.3.5 Correlation between milk nutrient intakes and plasma metabolite levels

As piglets were fed different volumes of milk in their last meal to match protein intake (2 g/kg BW; Table 3-2), this adjustment resulted in different amounts of nutrients consumed by piglets between milk groups (Table 3-1**,** Table 3-3). Hence, the analysis was performed to correlate milk nutrient intakes with plasma metabolite concentration, regardless of milk treatments.

Several significant correlations ($P < 0.05$) between milk amino acid intakes of the last meal (Error! Reference source not found.) and plasma amino acid concentrations (Table 3-5) were observed (Figure 3-5). For instance, plasma tyrosine was positively correlated with alanine (rho = 0.50), arginine (rho = 0.49), and tyrosine (rho = 0.45) intakes while negatively correlated (rho= -0.41) with threonine intake.

Significant relationships between milk lipid intakes (Table 3-3) and plasma lipid relative intensities (Table 3-6) were also observed (Figure 3-6). For instance, plasma SM 32:1,2O was negatively correlated with milk TG.26.0, TG.28.0, TG.30.0, TG.39.2, TG.41.2, TG.42.3, TG.43.1, TG.43.2, TG.46.3, TG.49.3, TG.53.4, TG.56.6 (rho range -0.43 to -0.69) intakes.

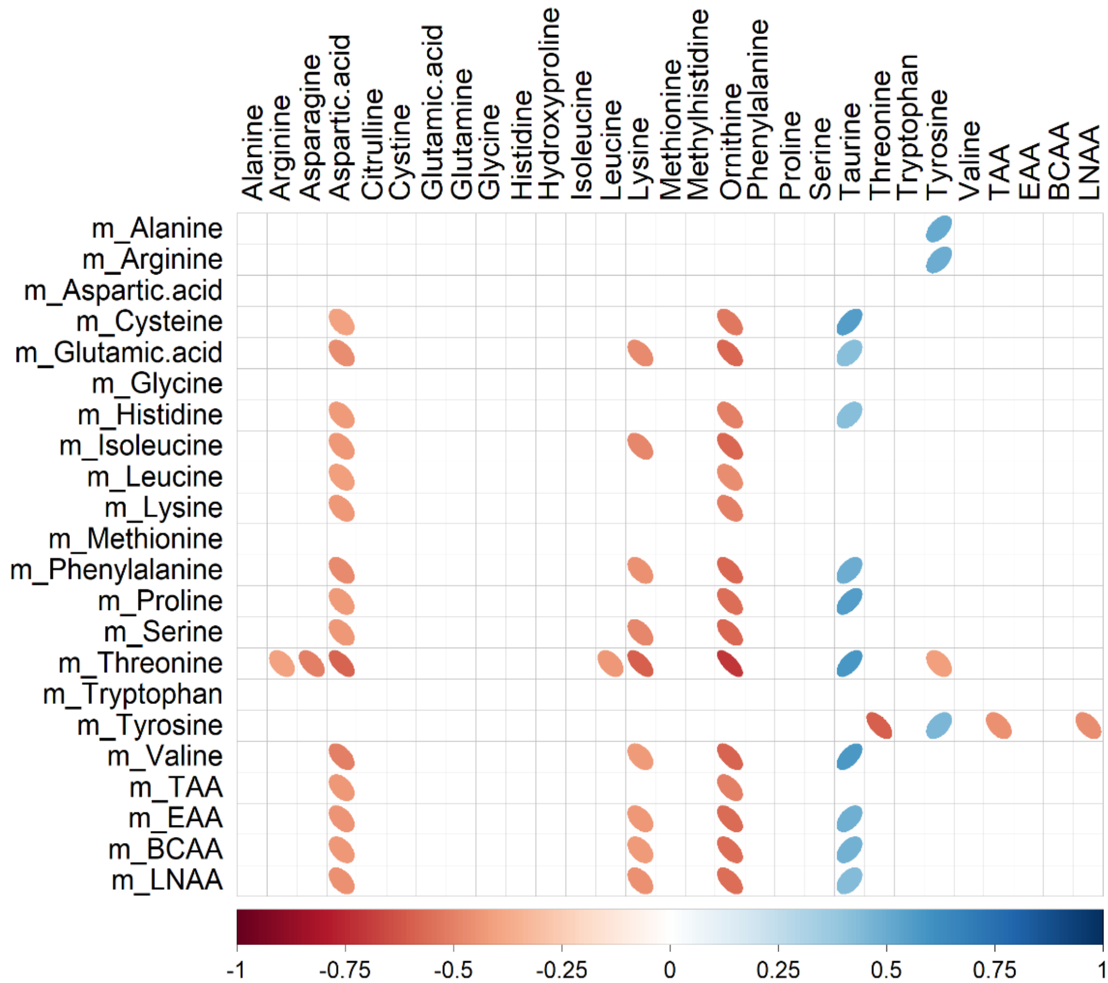
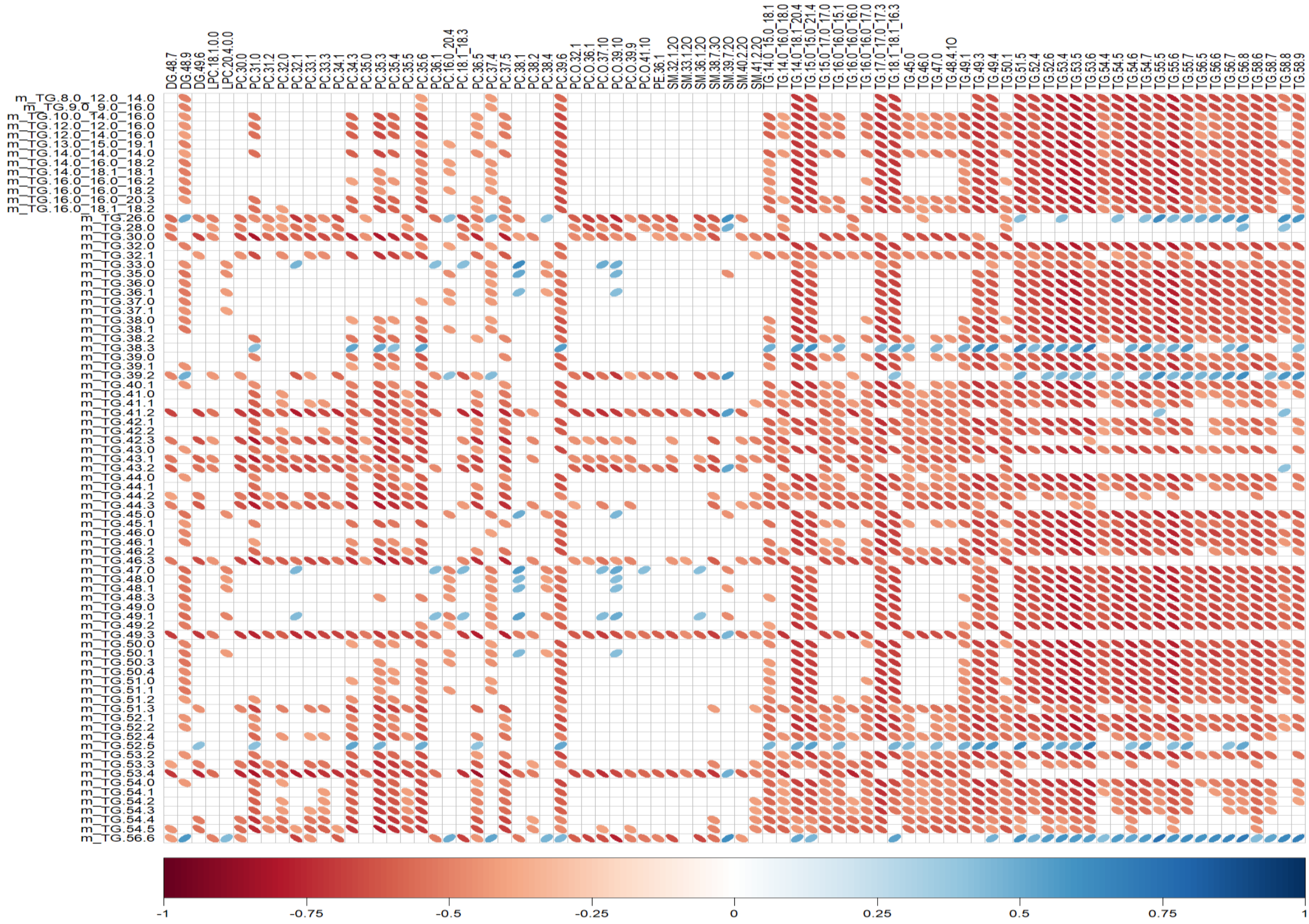



**Figure 3-5 Correlation plot showing Spearman correlations between milk amino acid intake in the last meal (mg) and plasma amino acid concentrations (mg/mL) of piglets fed bovine, caprine, or ovine milk treatment.**

The colour of the ellipse indicates the type of correlation, i.e., blue indicates a positive correlation, and red indicates a negative correlation. The ellipse shape indicates the magnitude of the correlation, i.e., the stronger the correlation, the flatter the ellipse. Only significant correlations ($P < 0.05$) are shown. The legend at the bottom of each correlation plot shows the correlation coefficients with their corresponding colours. The vertical axis represents amino acid intakes (preceded with the letter m), and the horizontal axis represents plasma amino acid concentrations .



DG.48.7
DG.48.9
DG.49.6
LPC.18.1.0.0
LPC.20.4.0.0
PC.30.0
PC.31.0
PC.31.2
PC.32.0
PC.32.1
PC.33.1
PC.33.3
PC.34.1
PC.34.3
PC.35.0
PC.35.3
PC.35.4
PC.35.5
PC.35.6
PC.36.1
PC.16.0_20.4
PC.18.1_18.3
PC.36.5
PC.37.4
PC.37.5
PC.38.1
PC.38.2
PC.38.4
PC.39.6
PC.O.32.1
PC.O.36.1
PC.O.37.10
PC.O.39.10
PC.O.39.9
PC.O.41.10
PE.36.1
SM.32.1.2O
SM.33.1.2O
SM.36.1.2O
SM.38.7.3O
SM.39.7.2O
SM.40.2.2O
SM.41.2.2O
TG.14.0_15.0_18.1
TG.14.0_16.0_18.0
TG.14.0_18.1_20.4
TG.15.0_15.0_21.4
TG.15.0_17.0_17.0
TG.16.0_16.0_15.1
TG.16.0_16.0_16.0
TG.16.0_16.0_17.0
TG.17.0_17.0_17.3
TG.18.1_18.1_16.3
TG.45.0
TG.46.0
TG.47.0
TG.48.4.1O
TG.49.1
TG.49.3
TG.49.4
TG.50.1
TG.51.5
TG.52.4
TG.52.6
TG.53.4
TG.53.5
TG.53.6
TG.54.4
TG.54.5
TG.54.6
TG.54.7
TG.55.5
TG.55.6
TG.55.7
TG.56.5
TG.56.6
TG.56.7
TG.56.8
TG.58.6
TG.58.7
TG.58.8
TG.58.9
m_TG.8.0_12.0_14.0
m_TG.9.0_9.0_16.0
m_TG.10.0_14.0_16.0
m_TG.12.0_12.0_16.0
m_TG.12.0_14.0_16.0
m_TG.13.0_15.0_19.1
m_TG.14.0_14.0_14.0
m_TG.14.0_16.0_18.2
m_TG.14.0_18.1_18.1
m_TG.16.0_16.0_16.2
m_TG.16.0_16.0_18.2
m_TG.16.0_16.0_20.3
m_TG.16.0_18.1_18.2
m_TG.26.0
m_TG.28.0
m_TG.30.0
m_TG.32.0
m_TG.32.1
m_TG.33.0
m_TG.35.0
m_TG.36.0
m_TG.36.1
m_TG.37.0
m_TG.37.1
m_TG.38.0
m_TG.38.1
m_TG.38.2
m_TG.38.3
m_TG.39.0
m_TG.39.1
m_TG.39.2
m_TG.40.1
m_TG.41.0
m_TG.41.1
m_TG.41.2
m_TG.42.1
m_TG.42.2
m_TG.42.3
m_TG.43.0
m_TG.43.1
m_TG.43.2
m_TG.44.0
m_TG.44.1
m_TG.44.2
m_TG.44.3
m_TG.45.0
m_TG.45.1
m_TG.46.0
m_TG.46.1
m_TG.46.2
m_TG.46.3
m_TG.47.0
m_TG.48.0
m_TG.48.1
m_TG.48.3
m_TG.49.0
m_TG.49.1
m_TG.49.2
m_TG.49.3
m_TG.50.0
m_TG.50.1
m_TG.50.3
m_TG.50.4
m_TG.51.0
m_TG.51.1
m_TG.51.2
m_TG.51.3
m_TG.52.1
m_TG.52.2
m_TG.52.4
m_TG.52.5
m_TG.53.2
m_TG.53.3
m_TG.53.4
m_TG.54.0
m_TG.54.1
m_TG.54.2
m_TG.54.3
m_TG.54.4
m_TG.54.5
m_TG.56.6
-1
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1

**Figure 3-6 Correlation plot showing Spearman correlations between milk lipid intakes (mg) and plasma lipid relative intensities of piglets fed bovine, caprine, or ovine milk treatment.**

The colour of the ellipse indicates the type of correlation, i.e., blue indicates a positive correlation, and red indicates a negative correlation. The ellipse shape indicates the magnitude of the correlation, i.e., the stronger the correlation, the flatter the ellipse. Only significant correlations ($P < 0.05$) are shown. The legend at the bottom of each correlation plot shows the correlation coefficients with their corresponding colours. The vertical axis represents milk lipid intakes (preceded with the letter m), and the horizontal axis represents plasma lipid relative intensities.

## 3.4 Discussion

This study is the first to explore the effects of feeding whole milk from bovine, caprine or ovine species on plasma polar and non-polar (lipids) metabolites using an LC-MS-based metabolomics approach in young piglets as a model of the human infant. The result showed that plasma polar and lipid metabolites changed in response to ruminant milk treatments. In total, 90 polar and lipid metabolites with differential relative intensities in plasma samples discriminated bovine, caprine, and ovine milk treatments, and some of these metabolites were correlated with milk nutrient intakes in the last meal.

### 3.4.1 Plasma amino acid differences between milk treatments

Differences in relative intensities of polar plasma metabolites, predominantly those involved in amino acid metabolism, were observed between milk treatments. Similar results were observed when plasma amino acid concentrations were quantified. The intake of ovine milk led to greater relative intensities of proline, tyrosine, and isoleucine and amino acid derivatives (acetylcarnitine, phenyl-pyruvic acid) and concentrations of asparagine, leucine, and lysine in plasma than bovine and caprine milk treatments even though protein intakes were similar. Supporting this observation, a study in women aged 20-40 years showed that ovine milk consumption also resulted in higher postprandial plasma concentrations of leucine, lysine, and proline compared to bovine milk (Milan et al., 2020). However, unlike the present study, the authors did not balance the milk intake per g protein/kg BW and provided the same volume of milk to all the participants. Therefore, it could be expected that the ovine milk group would have had higher amino acids in the peripheral circulation, as it has a higher protein concentration than the other milk.

In the present study, piglets received similar amounts of amino acids in their last meal due to balancing the protein. Correlation analysis between amino acid intake and plasma amino acid appearance showed that only tyrosine intake was positively correlated to its plasma appearance. Hence, elevated levels of plasma amino acids in response to consuming ovine milk may be due to increased and/or more rapid amino acid absorption. This hypothesis is supported by a faster rate of gastric protein emptying in the piglets consuming whole raw ovine milk than in piglets fed whole raw caprine or bovine milk (Roy et al., 2022a). Additionally, a positive relationship between gastric protein emptying rate and duodenal and jejunal amino acid absorption rates has been reported in piglets (Montoya et al., 2018). However, in this study, the circulatory appearance of many amino acids was not different between milk treatments. This finding may be attributed to most amino acids (e.g., glutamine, Trp) in the diet being taken up by first-pass metabolism by splanchnic tissues (Stoll and Burrin, 2006; Van De Poll et al., 2007; Fernstrom, 2016). Thus, any differences in amino acid profile may only be detected in the gut or liver may not be evident in the peripheral circulation.

The increase levels of some plasma amino acids observed in response to the ovine milk treatment could influence processes such as protein synthesis and degradation, hormone synthesis, and neurotransmission. For instance, proline is involved in glutamate neurotransmitter synthesis, whereas tyrosine is involved in dopamine and norepinephrine synthesis (Bertolo and Burrin, 2008; Cappelletti et al., 2018; Fernstrom and Fernstrom, 2018). Hence, it is plausible that increased concentrations of some amino acids in the peripheral circulation would increase neurotransmitter production in the gut, likely influencing motility and secretion. (Schwarz et al., 2001; Li, 2006) and/or brain sensory information, learning, and memory. (McEntee and Crook, 1993; Hasselmo, 2006).

### 3.4.2 Plasma lipid differences between milk treatments

Differences in plasma TG, PC, SM, LPC and DG relative intensities were observed between milk treatments. Piglets fed bovine milk had higher relative intensities of plasma lipids, except for unsaturated TG, than those fed ovine or caprine milk. A study in piglets of PND28 fed bovine or caprine milk showed that their plasma lipid profiles had predominantly TG, PC, SM, DG, and ceramide (CER) (Wang and Zhu, 2021). However, the authors did not compare plasma lipids between ruminant milk treatments, but they compared the plasma lipid profile of bovine or caprine milk with human breast milk and showed differences in the lipid profiles.

In the present study, plasma saturated and unsaturated TG relative intensities differed between milk treatments. Piglets fed ovine milk showed higher relative intensities of plasma unsaturated TG than that of caprine or bovine milk, possibly due to faster gastric lipid emptying (Roy et al., 2022). In addition, studies in rats showed that unsaturated fatty acids were more efficiently absorbed than saturated fatty acids in the small intestine (Gallagher and Playoust, 1969; Ockner et al., 1972), supporting the appearance of higher levels of unsaturated TG in the peripheral circulation following consumption of ovine milk. On the contrary, piglets fed bovine milk showed higher relative intensities of plasma saturated TG than that of caprine or ovine milk might be due to the higher saturated TG intake of the bovine milk-fed piglets. Furthermore, studies have shown that absorption of TG is efficient when lipids intake is high (Kasper, 1970; Mansbach and Dowell, 2000), supporting higher levels of saturated TG in the plasma of piglets fed with bovine milk.

However, negative correlations were observed between TG intake and its appearance in circulation in the current study. The complex digestion and absorption patterns of lipids might explain this. For example, TG undergoes hydrolysis of fatty acids and

monoacylglycerol in the small intestinal lumen; these metabolites then enter enterocytes and are incorporated into TG, which are packaged into chylomicrons transported in the lymph circulation to the systemic circulation (Yen et al., 2015). Therefore, the lipid species ingested might have been broken down and assembled into different lipid species that appeared in the peripheral circulation. It is important to note that due to the nature of the milk lipid data (relative intensity data), it was challenging to determine the exact intake of lipids in the milk. Hence, the milk lipid intake data presented in the current study might not accurately represent the actual lipids intakes of the piglets.

The changes in relative intensities of lipids in the peripheral circulation could influence lipid synthesis, cell signalling, and the structural components of cell membranes. For example, phospholipids are precursors of the neurotransmitter acetylcholine, (Jope and Jenden, 1979; Lopez et al., 1991) which regulates gut motility and secretions, learning, and memory (Tanaka et al., 2002; Haam and Yakel, 2017; Maurer and Williams, 2017). Peripheral circulating LPC, produced from the hydrolysis of phospholipid catalysed by acyltransferases and phospholipase, serve as a transporter for long polyunsaturated fatty acids to the brain, ultimately regulating central cell signalling and cell membrane remodelling (Semba, 2020; Liu et al., 2021). Isotope labelling dietary lipid molecules could help understand their appearance in the blood, and future studies should ascertain the implications of changes in plasma lipids in early postnatal life development.

### 3.4.3 Strengths and limitations

The main strength of this study was the application of metabolomics for analysing plasma metabolites. This approach provided a comprehensive analysis of measurable metabolites in a biological sample, which otherwise would have been missed with the targeted approach that detects only specific metabolites. Furthermore, using the LC-MS analytical

platform is another strength of this study which offered high sensitivity and detection of many metabolites compared to other analytical platforms like nuclear magnetic resonance.

Another advantage of this study is using piglets as a model for human infants to understand the plasma metabolome changes in response to different milk treatments. Furthermore, considering the higher similarity between piglets and humans in terms of gut and brain physiology, anatomy, and development than in other non-primate models like rodents (Guilloteau et al., 2010; Mudd and Dilger, 2017), the results can be translated for future studies with human infants, hence increasing applicability.

There are also some limitations which should be considered in interpreting findings. The use of metabolomics helped the detection of many plasma metabolites that were significant between milk treatments. However, most features remain unidentified. Additionally, LC-MS based metabolomics approach does not provide absolute quantification of metabolites, unlike NMR. No control groups of pigs fed with sow's milk showing basal expected plasma metabolic profile were compared with the pigs fed with other species' milk. This was because biological samples were collected from a study aimed to compare structural changes in ruminants' whole milk on pigs' digestion in early postnatal life, and using pigs fed with sow's milk was not required for the experiment.

Another limitation of this study was the use of a single time-point for evaluating the peripheral blood plasma metabolome profile in response to milk treatments, making it difficult to ascertain whether the observed changes were an immediate diet effect or an early postnatal developmental effect. Furthermore, only male piglets were used to understand the changes in plasma metabolite relative intensities. However, a study has shown that the plasma metabolome of children with autism spectrum disorder was sex-

specific (Sotelo-Orozco et al., 2020). Hence, a comparative analysis of the plasma metabolome between male and female piglets would have been informative, but it would have required more piglets per treatment.

### 3.4.4 Conclusion

This study compared the consumption of bovine, caprine, and ovine whole milk on circulatory plasma polar and non-polar metabolite profiles in piglets (as a model of the human infant) using LC-MS-metabolomics. Plasma metabolites, predominantly lipids, were altered between milk treatments. Relative intensities of unsaturated TG were higher in response to the ovine milk treatment, whereas saturated TG was higher in response to the bovine milk treatment. The potential implications of ruminant milk-associated circulating metabolite profile for brain metabolite uptake and metabolism in the early postnatal period remain to be elucidated.

# Chapter 4

# Metabolite profiling of cognitive areas of the brain in response to ruminants’ milk in early postnatal life

## Abstract

Breast milk is recognised to play an important role in an infant's brain development. Whether milk from different ruminant species influences the brain metabolite important for the developing brain differently remains unknown. This study aimed to determine the effects of exclusively feeding raw whole bovine, caprine, or ovine milk on relative intensities of polar and non-polar (lipid) metabolites of brain areas associated with cognitive function (hippocampus, prefrontal cortex, and striatum) in entire male piglets from PND ~7 to ~21, as a model of human infants. A secondary aim was to compare the metabolite profiles between these brain regions, regardless of the milk treatments. The relative intensities of metabolites were measured using LCMS-based metabolomics. Polar and lipid metabolites differed in their relative intensities between brain regions (particularly the striatum), regardless of the milk treatments. Significant differences in the relative intensity of brain lipids (mostly glycerophospholipids) were observed between milk treatments, with the striatum being the most affected (44 lipids), followed by the prefrontal cortex (5) and hippocampus (5). The piglets fed bovine milk had higher brain lipid profiles linked to predicted activated lipid biosynthesis pathways compared to those fed other milk treatments. The relative intensities of the polar metabolite, threonine, in the hippocampus and striatum were higher in the caprine milk treatment compared to the other milk treatments. This study presented the first evidence that consuming whole bovine, caprine, or ovine milk influences specific metabolites (mainly lipids) that may subsequently modulate early postnatal brain development in piglets.

## 4.1 Introduction

While the development of the brain begins *in utero*, the brain continues to develop in the early postnatal years of life. During that period, brain developmental events involve the rapid growth of regional brain volumes, refinement of neural connections, including synaptic pruning, neurotransmitter function, myelination and neurite outgrowth, and the establishment of cognitive abilities (Tau and Peterson, 2009; Gilmore et al., 2012, 2018). Moreover, given its responsiveness toward environmental factors (e.g., early postnatal life nutrition, caregiving) (Nelson et al., 2007; Mudd et al., 2016), any stimuli or insult during this period could optimise or diminish brain development. Hence, the adaptability of the developing brain makes this period critical for shaping behavioural and health outcomes in adulthood.

Human breast milk is the gold standard for infant nutrition. However, when breast milk is unavailable or limited, formula tailored to provide nutrients for infant growth and development is an alternative or complementary to human breast milk. Infant formula is most commonly bovine milk-based, but milk from ovine and caprine species is also increasingly used for making infant formula.

Beyond its nutritional benefits, milk is increasingly recognised for its potential to influence brain development postnatally (Lin et al., 2019). For instance, studies in young piglets have shown that feeding bovine milk osteopontin, a whey protein and oligosaccharide with sialyllactose, improved spatial cognition, increased gene expression in the hippocampus related to sialic acid metabolism, myelination, and ganglioside biosynthesis and supported neurodevelopment (Obelitz-Ryom et al., 2019; Joung et al., 2020).

However, the nutrient composition of milk used to make formula differs between bovine, caprine, or ovine milk (Park et al., 2007; Claeys et al., 2014; Rafiq et al., 2016; Felice et al., 2021). For instance, ovine milk contains more lipids, proteins, and energy than caprine or bovine milk (Barlowska et al., 2011; Claeys et al., 2014). Caprine milk has higher levels and diversity of oligosaccharides than bovine and ovine milk (Van Leeuwen et al., 2020; Shi et al., 2021). Ovine and caprine milk contain higher levels of small and medium-chain TG than bovine milk (Ruiz-Sala et al., 1996). In addition, ovine milk has higher levels of essential amino acids than bovine and caprine milk (Rafiq et al. 2016; Claeys et al. 2014).

Recent findings showed that the gastric emptying rate of proteins and lipids from ovine and caprine milk in piglets was faster compared to bovine milk (Roy et al., 2022a). These findings suggest that consuming ovine or caprine milk may increase amino acids and lipids available for small intestinal absorption, resulting in more metabolites appearing in the blood and at the BBB and potentially affecting brain metabolism and function. Supporting this, feeding a diet enriched in milk gangliosides and phospholipids to male rats from PND 10 to 80 elicited greater tyrosine hydroxylase staining in the striatum, indicating elevated dopamine output and synaptophysin density in the hippocampus (Guillermo et al., 2015). Both outcomes were associated with enhanced learning and memory (Ya et al., 2013; Guan et al., 2015). However, there was no blood or brain metabolite profile measurement in these rats. Another recent study highlighted that feeding infant formula supplemented with high levels of MFGM increased TG species relative intensities in the hippocampus of neonatal piglets compared to feeding formula with low levels milk fat globule membrane (Fraser et al., 2022).

Metabolites in the brain play a role in cell signalling, neural membrane structure, receptor membrane protein functions, synaptic plasticity, and memory formation. However,

whether consuming bovine, caprine and ovine milk translates into changes in brain metabolic pathways involved in cognitive development remains unknown.

Based on the existing evidence, it was hypothesised that bovine, caprine or ovine milk with different nutrient compositions and gastric emptying rates would influence the relative intensities of the metabolites in brain areas related to cognitive development. Therefore, the aim was to determine the effects of these milk treatments on the metabolome of the hippocampus, striatum, and prefrontal cortex in piglets as a model of human infants. In addition, the metabolite profiles of these brain areas were compared, regardless of the milk treatments.

# 4.2 Materials and methods

## 4.2.1 Animal study

The study design was described in Chapter 3 (c.f., section 3.2.2).

## 4.2.2 Brain tissue sampling

The piglets were anaesthetised using a Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL, Zoetis Inc.) reconstituted with 2.5 mL each of ketamine and xylazine (both 100 mg/mL). The solution containing 50 mg/mL of each drug was administered at a dose rate of 0.4 mL/10 kg of BW by intramuscular injection, followed by euthanisation using a lethal dose (0.3 mL/kg BW) of pentobarbitone (Pentobarb 300, Provet NZ Pty Limited). The brain was carefully removed from the skull and immediately dissected on an ice-chilled surface, and the prefrontal cortex, hippocampus, and striatum were collected from the left hemisphere only for consistency with other studies (Zhang et al., 2016; Negi and Guda, 2017). The dissected brain regions were snap-frozen in liquid nitrogen and stored in a -80 °C freezer. Brain tissue samples were collected from twenty-three piglets, as one brain tissue sample was not collected during sampling days.

### 4.2.3 Metabolomics analysis

#### 4.2.3.1 Chemicals

Chemicals and solvents used were LC-MS grade unless specified. Acetonitrile, isopropanol, methanol, chloroform (analytical grade), and formic acid were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium formate (HPLC grade) and internal standards ($d_5$-Trp, $d_{10}$-leucine, $d_2$-tyrosine, and $d_7$-alanine) used in extraction solvent were obtained from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q® ultrapure water was obtained from Merck Millipore (Bedford, MA, USA). SPLASH® lipidomix® MS standard containing all the major lipid classes was obtained from Avanti® (Alabaster, AL, USA).

#### 4.2.3.2 Sample preparation

Extraction solvent (chloroform: methanol 1:1 v/v containing internal standards $d_5$- Trp, $d_{10}$-leucine, $d_2$-tyrosine, and $d_7$-alanine) of 800 µL precooled at -20°C was added to each 2 mL microcentrifuge tubes prefilled with 50 mg of brain sample and 3 mm zirconium oxide beads, and homogenised using a Tissue Lyser II (Qiagen, Hilden, Germany) for 2 min at 30 Hz. The homogenised samples were vortexed for 30 s and then incubated for 60 min at -20°C. Subsequently, 400 µL of Milli-Q® water was added to each sample, vortexed for 30 s and then centrifuged for 10 min at 11,000 rpm at 4°C. Thereafter, 200 µL aliquots of the supernatant and 200 µL of the bottom layer were transferred into new 2 mL microcentrifuge tubes for polar (upper layer) and non-polar (bottom) metabolite analyses. Pooled polar quality control samples were prepared by combining 40 µL of the supernatant from each sample into a new tube, vortexed for 30 s, and then aliquoted into multiple microcentrifuge tubes. Similar procedures were followed for non-polar quality control samples, except the aliquots were taken from the bottom layer. Blank samples were prepared using the above procedures, except the samples were replaced with Milli-

Q® water. The microcentrifuge tubes containing samples, quality control and blank were dried under a stream of nitrogen at room temperature and stored at -80°C. On the day of LC-MS analysis, dried polar and non-polar extracts were reconstituted in 300 µL of acetonitrile: water (1:1 v/v) containing formic acid (0.1 %), 800 µL of chloroform: methanol (2:1 v/v) and SPLASH® mix, containing all the major lipid classes for polar and non-polar metabolite analysis, respectively.

### 4.2.3.3 Liquid chromatography-mass spectrometry analysis

Metabolites were analysed using a LCMS-9030 mass spectrometer coupled with a Nexera-x2 ultra performance liquid chromatography system (Shimadzu, Kyoto, Kyoto, Japan) as described in full in Chapter 3 (c.f., 3.2.5.3).

Briefly, chromatographic separations of polar and non-polar metabolites were conducted by injecting 5 µL of samples onto Accucore™ HILIC column, 2.1 mm × 100 mm, 2.6 µm particle size (Thermo Fisher Scientific, Waltham, MA, USA), and 2 µL of samples onto the CSH-C18 column, 2.1 × 100 mm, 1.7 µm particle size (Waters, Milford, MA, USA), respectively. The mobile phases used for chromatographic separations were 10 mM ammonium formate in water (solvent A) and 0.1 % of formic acid in acetonitrile (solvent B) for polar metabolites and 10 mM of ammonium formate in water/acetonitrile/isopropanol (5:3:2 v/v) (solvent A) and 10 mM of ammonium formate in water/acetonitrile/isopropanol (1:9:90 v/v) (solvent B) for non-polar metabolites. The mass spectral detection for polar metabolites was performed in positive and negative ionisation modes. In contrast, for non-polar metabolites, mass spectral detection was only performed in positive ionisation mode, as it captures most lipids.

#### 4.2.3.4 Data processing

Raw data files were processed using the same steps described in Chapter 3 (c.f., 3.2.5.4), except for the parameters used for processing. For polar metabolite data, analysed in positive and negative ionisation modes, minimum peak height was 2,000 and 1,000, respectively, and retention time tolerance was 0.3 min. For non-polar metabolite data analysed in positive ionisation mode, the minimum peak height was 3,000, and the retention time tolerance was 0.15 min.

### 4.2.4 Statistical analyses

Multivariate statistical analyses, including PCA and PLS-DA, were conducted to investigate differences in polar and non-polar metabolite profiles between milk treatments and between brain regions using SIMCA (version 16). Briefly, PCA analysed the overall variation in the samples, whereas PLS-DA discriminated the samples based on class information.

The PLS-DA models were validated using the predictive ability of the model ($Q^2 > 0.5$) and CV-ANOVA (P value < 0.05) cut-offs of a good model for multivariate data. Permutation tests involving 100 permutations were used to check the robustness of the model. Using the PLS-DA model, variables that contributed the most to separating intensities between treatments were selected (VIP > 1). A one-way ANOVA was conducted using the Metaboanalyst platform (version 5.0) (Pang et al., 2021), and the relative intensities of metabolites with a FDR < 0.05 were deemed significant. A pair-wise FC of relative intensities of metabolites was significant between milk treatments or between brain regions with an FDR < 0.05 and $Log_2FC > 1$ (higher relative intensity) or $Log_2FC < -1$ (lower relative intensity) (equivalent to FC > 2)

The lipidome data was visualised using BioPAN, a web-based software available on the LIPIDMAPS® Lipidomics Gateway in the context of known biosynthetic pathways (Lopez-Clavijo et al., 2021). Brain metabolite profiles of piglets fed different milk treatments were compared in pairs to estimate the activated or suppressed status of metabolic pathways. If the product of a metabolic pathway was higher in one milk treatment than in another milk treatment, then the pathway was likely more active, and if the product of a reaction was lower among these comparisons, then this pathway was likely suppressed. A pathway with P value < 0.05 (corresponding Z-score > 1.645) identified using a t-test was considered significant.

The association between milk lipid intake (average daily lipid intake) (Appendix Table A3) and brain lipid metabolite relative intensities, irrespective of the milk treatments, were assessed using Spearman correlations. The average daily intake was calculated from PND 9 or 10 onwards, as the first three experimental days of the acclimatisation phase had a substantial amount of milk spills and refusals. Average daily lipid intake was used in the analysis as changes in the brain's lipid metabolite profile reflect the continuous turnover of metabolites over a period. Spearman rank correlation coefficient and the corresponding P-value were calculated using the cor. test function and visualised using the corrplot package in R. Correlations with P < 0.05 and rho > 0.5 or rho < -0.5 were considered significant.

## 4.3 Results

The numbers of features of polar metabolites obtained in positive and negative ionisation modes were 480 and 405, respectively. After filtration and background noise removal, positive and negative ionisation data were combined into one data set. The resulting 199 variables (44 known metabolites and 157 unknown features) were used for multivariate

analyses. Lipid metabolite profile data (662 features) were obtained only in positive ionisation mode. After filtration and removal of background noise, 169 features were identified and used for multivariate analyses.

First, the data were analysed to determine if there were different relative intensities of metabolites between brain areas, regardless of milk treatments. Then, the effects of milk treatments on these parameters were compared within each brain region.

## 4.3.1 Brain region effect

### 4.3.1.1 Polar metabolites

The PCA plot showed that polar metabolites differed between brain regions (Appendix Figure A2A). Three hippocampus samples (from three different milk treatment groups) were identified as outliers in the PCA plot, most likely due to an error in the LC-MS sample injection, and they were removed. A validated PLS-DA model (Q2 = 0.91 and CV-ANOVA P value < 0.05) with a good fit also showed a clear and robust separation of polar metabolites between brain regions (Figure 4-1A-B).

Eighty-eight polar metabolites had significantly (FDR < 0.05) different relative intensities between brain regions, as well as contributed the most to the separation between brain regions (VIP > 1), of which 20 metabolites were identifiable (Table 4-1). These polar metabolites were selected for pair-wise comparisons between brain areas. The relative intensity of citrate was higher ($log_2FC > 1$ & FDR < 0.05), whereas that of L-carnitine and proline was lower ($log_2FC < -1$ & FDR < 0.05) in the hippocampus compared to the prefrontal cortex (Table 4-1). In the striatum, the relative intensity of cytidine and 3-methyl histidine was lower compared to the prefrontal cortex. In the hippocampus, the relative intensity of cytidine was higher, whereas L-carnitine decreased compared to the striatum.

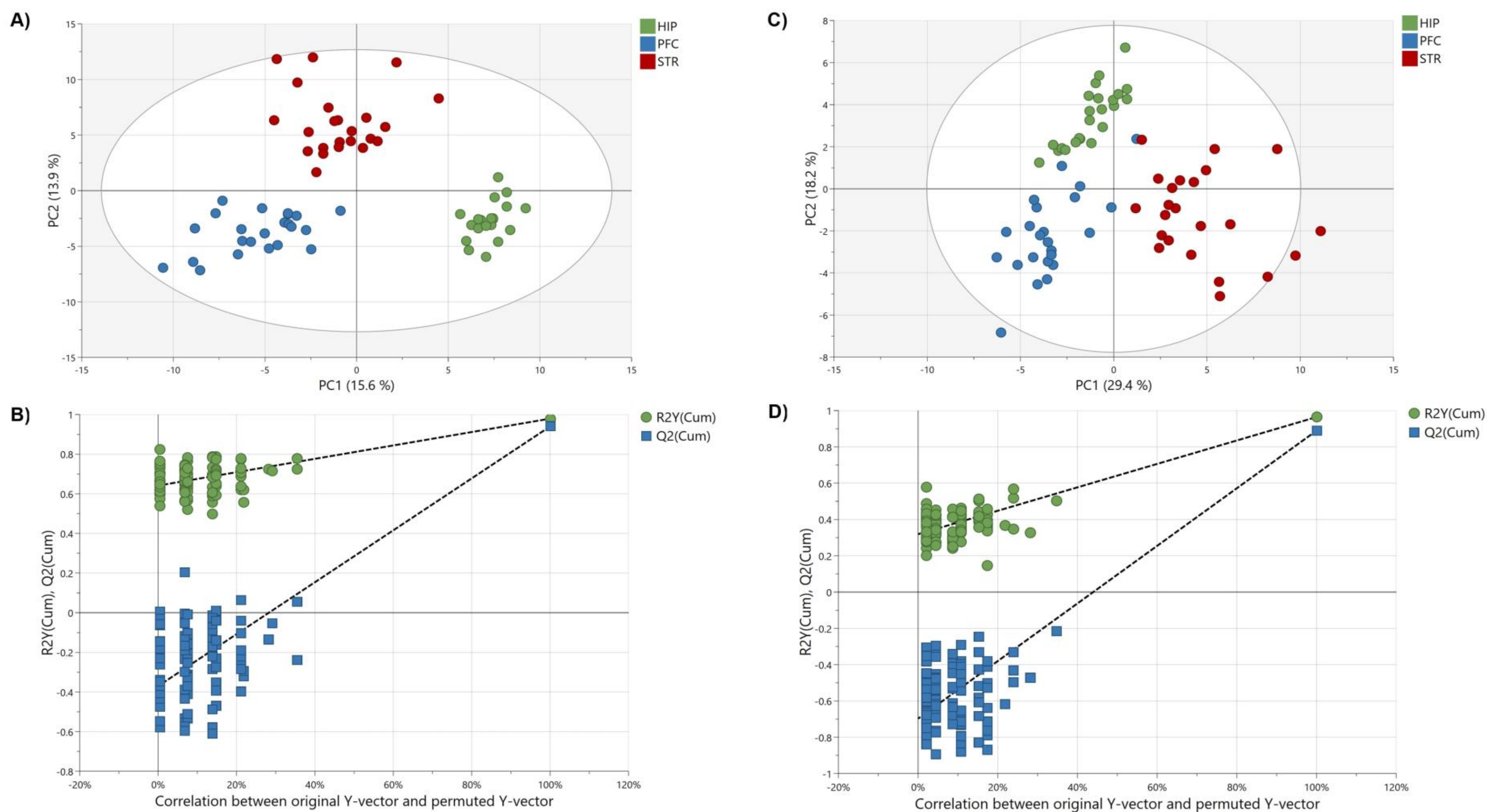



**Figure 4-1 Partial least squares discriminant analysis (PLS-DA) of metabolite relative intensity differences between the hippocampus (HIP), prefrontal cortex (PFC), and striatum (STR) of piglets exclusively fed whole bovine, caprine, or ovine milk treatment for 15 days.**

A) Score plot of polar metabolites; B) Permutation plot of polar metabolites; C) Score plot of non-polar (lipid) metabolites; D) Permutation plot of non-polar (lipid) metabolites. Permutation plots involve 100 permutation tests showing no overfitting of the PLS-DA model, confirming the robustness of the

model. The criteria for evaluating whether there is overfitting of the PLS-DA model is that Q2 and R2 values of the permutated Y model to the left were lower than the Q2 and R2 value of the original model to the far right. The first two principal components are plotted. Percentages of variation explained by each principal component are indicated along the axes. PC, principal component.

**Table 4-1 Brain polar metabolites with a significant difference in relative intensities between the hippocampus (n=20), prefrontal cortex (n=23), and striatum (n= 23) of piglets exclusively fed whole bovine, caprine or ovine milk treatment for 15 days⁺**

| Polar metabolites | VIP | FDR | $Log_2$fold change | | |
|---|---|---|---|---|---|
| | | | HIP vs PFC | STR vs PFC | HIP vs STR |
| Adenine | 1.43 | <0.01 | 0.01 | -0.72 | 0.73 |
| Choline | 1.15 | <0.01 | -0.19 | 0.03 | -0.22 |
| Citrate | 1.10 | <0.01 | 1.25* | 0.77 | 0.48 |
| Creatine | 1.24 | <0.01 | -0.14 | -0.09 | -0.05 |
| Creatinine | 1.38 | <0.01 | -0.24 | 0.18 | -0.42 |
| Cytidine | 1.23 | <0.01 | -0.52 | -1.56* | 1.04* |
| Gamma amino butyric acid | 1.47 | <0.01 | -0.06 | 0.37 | -0.44 |
| Isoleucine | 1.43 | <0.01 | -0.25 | -0.47 | 0.22 |
| L-5-Oxoproline | 1.03 | <0.01 | -0.17 | -0.27 | 0.11 |
| L-Carnitine | 1.50 | <0.01 | -1.73* | 0.14 | -1.87* |
| Leucine | 1.28 | <0.01 | -0.22 | -0.41 | 0.20 |
| L-Glutamine | 1.08 | <0.01 | -0.21 | -0.25 | 0.04 |
| L-Phenylalanine | 1.22 | <0.01 | -0.39 | -0.46 | 0.07 |
| Methionine | 1.25 | <0.01 | -0.73 | -0.65 | -0.08 |
| L-3-Methyl histidine | 1.15 | <0.01 | -0.30 | -1.07* | 0.77 |
| Proline | 1.11 | <0.01 | -1.05* | -0.19 | -0.86 |

| | | | | | |
|---|---|---|---|---|---|
| Taurine | 1.28 | <0.01 | -0.62 | -0.13 | -0.49 |
| Tyrosine | 1.30 | <0.01 | -0.34 | -0.64 | 0.30 |
| Uridine | 1.50 | <0.01 | 0.99 | 0.46 | 0.53 |
| Valine | 1.45 | <0.01 | -0.31 | 0.39 | -0.70 |

[+] *Variable importance in projection (VIP) >1 from the partial least squares discriminant analysis model was used to select the contributing polar metabolites. One-way ANOVA was used to determine the significant polar metabolites between brain areas (FDR < 0.05). Only polar metabolites that satisfied multivariate and ANOVA analysis criteria are shown. *Metabolites with significant $log_2$fold change > 1 or $log_2$fold change < -1 (false discovery rate (FDR) < 0.05, using t-test) between brain areas are shown. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum*

#### 4.3.1.2 Non-polar metabolites

The PCA plot showed lipid metabolite profiles differed between brain regions (Appendix FigureA2B). In addition, a validated PLS-DA model (Q2 = 0.9 and CV-ANOVA P value < 0.05) with a good fit also showed a clear and robust separation of lipids between brain regions (Figure 4-1C-D).

Forty-five lipids had significantly (FDR < 0.05) different relative intensities between brain regions, as well as contributed the most to the separation between brain regions (VIP > 1) (Table 4-2). Therefore, these lipids were selected for pair-wise comparisons between brain areas. The relative intensities of one CER 44:4;3O, two PC (38:2, O-32:1), and one phosphatidylethanolamine ((PE), P-38:5) were higher in the hippocampus than in the prefrontal cortex (Table 4-2). In the striatum, the relative intensities of five CER, one LPE 20:4, seven PC, and four PE were higher, whereas that of PC 32:2 was lower compared to the prefrontal cortex. In addition, the relative intensity of PC 32:2 was higher in the hippocampus, whereas two CER, two PC and three PE were lower in intensity than in the striatum.

**Table 4-2 Brain lipids with a significant difference in relative intensities between the hippocampus (n=20), prefrontal cortex (n=23), and striatum (n= 23) of piglets exclusively fed whole bovine, caprine or ovine milk treatment for 15 days[+]**

| Lipids | VIP | FDR | $Log_2$fold change | | |
|---|---|---|---|---|---|
| | | | **HIP vs PFC** | **STR vs PFC** | **HIP vs STR** |
| Cer 36:1;2O | 1.08 | <0.01 | 0.36 | 0.72 | -0.35 |
| Cer 38:1;2O | 1.02 | <0.01 | 0.82 | 1.15* | -0.33 |
| Cer 38:1;3O | 1.13 | <0.01 | 0.88 | 2.80* | -1.92 |
| Cer 38:3;3O | 1.24 | <0.01 | 0.38 | 2.39* | -2.01* |
| Cer 40:3;3O | 1.09 | <0.01 | -0.22 | 2.21* | -2.43* |
| Cer 44:4;3O | 1.08 | <0.01 | 1.17* | 2.11* | -0.93 |
| DG 37:7 | 1.06 | <0.01 | -0.20 | -0.41 | 0.21 |
| DG 41:6 | 1.12 | <0.01 | -0.07 | -0.44 | 0.36 |
| DG 45:6 | 1.01 | <0.01 | 0.50 | 0.53 | -0.03 |
| DG 45:7 | 1.07 | <0.01 | 0.15 | -0.16 | 0.31 |
| DG 46:9 | 1.07 | <0.01 | 0.54 | 0.01 | 0.53 |
| LPE 20:4 | 1.24 | <0.01 | 0.92 | 1.41* | -0.49 |
| PC 30:1 | 1.19 | <0.01 | 0.32 | -0.57 | 0.89 |
| PC 32:0 | 1.10 | <0.01 | 0.05 | 0.17 | -0.12 |
| PC 32:2 | 1.42 | <0.01 | -0.41 | -1.56* | 1.15* |
| PC 35:5 | 1.22 | 0.03 | -0.34 | 0.37 | -0.71 |

| | | | | | |
|---|---|---|---|---|---|
| PC 36:3 | 1.18 | 0.01 | -0.05 | -0.20 | 0.14 |
| PC 36:4 | 1.43 | <0.01 | 0.30 | 0.60 | -0.29 |
| PC 38:2 | 1.00 | <0.01 | 1.96* | 1.31* | 0.65 |
| PC 38:4 | 1.29 | <0.01 | 0.48 | 1.25* | -0.77 |
| PC 38:5 | 1.36 | <0.01 | 0.53 | 1.30* | -0.76 |
| PC 38:6 | 1.71 | <0.01 | 0.15 | 0.73 | -0.58 |
| PC 40:6 | 1.56 | <0.01 | 0.18 | 1.73* | -1.55* |
| PC 40:7 | 1.71 | <0.01 | 0.07 | 0.98 | -0.91 |
| PC 42:10 | 1.61 | <0.01 | -0.11 | 0.90 | -1.01 |
| PC O-32:1 | 1.27 | <0.01 | 1.18* | 1.37* | -0.19 |
| PC O-34:0 | 1.02 | <0.01 | 0.44 | 1.76* | -1.31 |
| PC O-37:10 | 1.11 | 0.01 | -0.15 | -0.20 | 0.05 |
| PC O-37:8 | 1.56 | <0.01 | 0.13 | 1.38* | -1.25* |
| PE 36:4 | 1.14 | <0.01 | -0.02 | 3.82* | -3.84* |
| PE 38:3 | 1.13 | <0.01 | -0.13 | -0.33 | 0.20 |
| PE 38:4 | 1.27 | <0.01 | 0.38 | -0.35 | 0.73 |
| PE 40:5 | 1.44 | 0.02 | -0.40 | -0.20 | -0.20 |
| PE 40:6 | 1.19 | <0.01 | 0.02 | 1.71* | -1.69* |
| PE P-36:4 | 1.87 | <0.01 | 0.31 | 1.73* | -1.42* |

| | | | | | |
|---|---|---|---|---|---|
| PE P-38:4 | 1.44 | <0.01 | 0.29 | 0.90 | -0.61 |
| PE P-38:5 | 1.11 | <0.01 | 1.44* | 2.66* | -1.23 |
| PE P-40:4 | 1.05 | <0.01 | 0.73 | 0.12 | 0.61 |
| SM 34:1;2O | 1.02 | <0.01 | 0.28 | 0.54 | -0.26 |
| SM 38:4;2O | 1.10 | <0.01 | 0.58 | 0.03 | 0.55 |
| TG 50:1 | 1.09 | 0.03 | -0.41 | -0.22 | -0.19 |
| TG 50:2 | 1.22 | <0.01 | -0.52 | -0.46 | -0.06 |
| TG 52:0 | 1.12 | <0.01 | -0.17 | -0.79 | 0.62 |
| TG 52:2 | 1.14 | <0.01 | -0.59 | -0.42 | -0.16 |
| TG 52:3 | 1.15 | <0.01 | -0.53 | -0.47 | -0.06 |

[+] *Variable importance in projection (VIP) >1 from the partial least squares discriminant analysis model was used to select the contributing lipids. One-way ANOVA was used to determine the significant lipids between brain areas (FDR < 0.05). Only lipids that satisfied multivariate and ANOVA analysis criteria are shown. *Lipids with significant log$_2$fold change >1 or log$_2$fold change > -1 (false discovery rate (FDR) < 0.05, using t-test) between brain areas are shown. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum; Cer, ceramides; DG, diglycerides; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TG, triglycerides*

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### 4.3.2 Milk treatment effect

Regardless of milk treatments, the comparisons of the brain areas showed significant differentiation in the relative intensity of metabolites between the hippocampus, prefrontal cortex, and striatum. Therefore, the effect of exclusively feeding whole bovine, caprine, or ovine milk to piglets for 15 days on the brain metabolite profiles was assessed for each region.

#### 4.3.2.1 Polar metabolites

The PCA plots of polar metabolites showed minor separation in the hippocampus between milk treatments (Appendix Figure A3A). However, in the prefrontal cortex and striatum, there was no separation of polar metabolites between milk treatments (Appendix Figure A3B-C). In contrast, validated PLS-DA models (CV-ANOVA P value $< 0.05$, $Q2 = 0.82$, hippocampus; $Q2 = 0.56$, prefrontal cortex; $Q2 = 0.56$, striatum) with a good fit also showed a clear and robust separation of polar metabolites between milk treatments (Figure 4-2A-F).

Of the identifiable polar metabolites, only threonine in the hippocampus and taurine and threonine in the striatum had significantly ($FDR < 0.05$) different relative intensities between milk treatments as well as contributing most to the separation between milk treatments ($VIP > 1$) (Table 4-3). Polar metabolites in the prefrontal cortex did not differ significantly ($FDR > 0.05$) (data not shown). The relative intensity FC of threonine in the hippocampus was lower in the ovine milk group compared to that of the caprine milk group, while in the hippocampus and striatum of the caprine milk group, threonine was higher compared to the bovine milk group (Table 4-3).

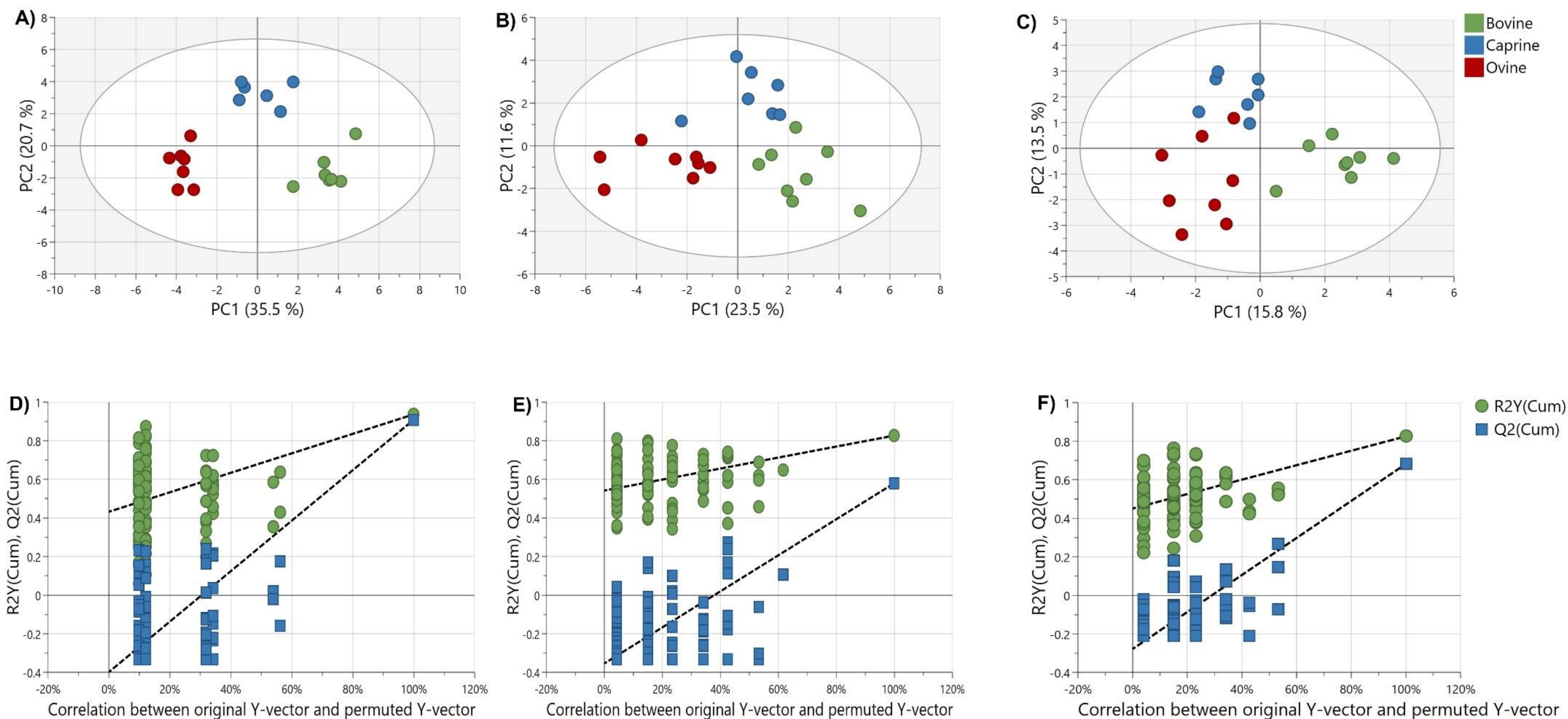


**Figure 4-2 Partial least squares discriminant analysis (PLS-DA) of polar metabolites relative intensity differences for brain tissue samples of piglets exclusively fed whole bovine, caprine, or ovine milk treatment for 15 days.**

Score plot of A) hippocampus, B) prefrontal cortex, C) and striatum. Permutation plot of D) hippocampal, E) prefrontal cortex, and F) striatal tissue. Permutation plots involve 100 permutation tests showing no overfitting of the PLS-DA model, confirming the robustness of the model. The criteria for evaluating whether there is overfitting of the PLS-DA model is that the Q2 and R2 values of the permutated Y model to the left were lower than the Q2 and R2 value of the original model to the far right. The first two principal components (PC) are plotted. Percentages of variation explained by each PC are indicated along the axes. PC, principal component. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum

**Table 4-3 Brain polar metabolites with a significant difference in relative intensity for brain tissue samples of piglets exclusively fed whole bovine, caprine, or ovine milk treatment for 15 days[+]**

| Region | Polar metabolites | VIP | FDR | $Log_2$fold change | | |
|---|---|---|---|---|---|---|
| | | | | Ovine vs Bovine | Ovine vs Caprine | Caprine vs Bovine |
| Hippocampus | Threonine | 1.67 | <0.01 | 0.08 | -1.12* | 1.21* |
| Striatum | Taurine | 2.5 | 0.04 | 0.30 | -0.18 | 0.48 |
| | Threonine | 2.12 | 0.02 | 0.25 | -0.98 | 1.23* |

[+] *Variable importance in projection (VIP) >1 from the partial least squares discriminant analysis model was used to select the contributing polar metabolites. One-way ANOVA was used to determine the significant polar metabolites between milk treatments (FDR < 0.05). Only polar metabolites that satisfied multivariate and ANOVA analysis criteria are shown. *Metabolites with significant $log_2$fold change > 1 or $log_2$fold change < -1 (false discovery rate (FDR) < 0.05, using t-test) between milk treatments are shown. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum*

#### 4.3.2.2 Nonpolar (lipid) metabolites

In the hippocampus, the PCA plot showed no separation of lipid metabolites between milk groups (Appendix Figure 4A). The PCA plots of lipid metabolites showed minor separation in the prefrontal cortex and striatum between milk groups; the ovine and caprine milk groups were more similar to each other than the bovine milk group (Appendix Figure A4 B-C). In contrast, validated PLS-DA models (CV-ANOVA P value < 0.05, Q2= 0.63, hippocampus; Q2= 0.45, prefrontal cortex; Q2= 0.82, striatum) with a good fit showed a clear and robust separation of lipid metabolites between milk treatments (Figure 4-3A-F).

Forty-four lipids in the striatum, five lipids in the prefrontal cortex and five lipids in the hippocampus differed (FDR < 0.05) in relative intensities between milk groups as well as contributed the most to the separation between milk treatments (VIP > 1) (Table 4-4). Therefore, these lipids were selected for pair-wise comparisons.

In the hippocampus, the relative intensity of phosphatidylserine ((PS) 40:5) was higher, whereas that of PS 36:1 was lower in the caprine group than in the bovine milk group (Table 4-4). In the prefrontal cortex, the relative intensity of PS 38:4 and PS 40:5 was lower in the bovine milk group than in the caprine and ovine milk groups. In the striatum of the bovine milk group, a higher relative intensity of 25 lipids (two CER, 15 glycerophospholipids (PC, PE, and PS), three SM, five TG) was observed compared to the ovine milk group, whereas 27 lipids (one CER, 17 glycerophospholipids (PC, PE, and PS), three SM, six TG) were higher in relative intensity compared to caprine milk group. Only the relative intensity of PS (38:4) in the striatum was higher in the ovine milk group compared to the caprine milk group (Table 4-4). There was no difference in relative intensity of lipid metabolites in the hippocampus or prefrontal cortex between ovine and

caprine milk groups or in the hippocampus between ovine and bovine milk groups (Table 4-4).

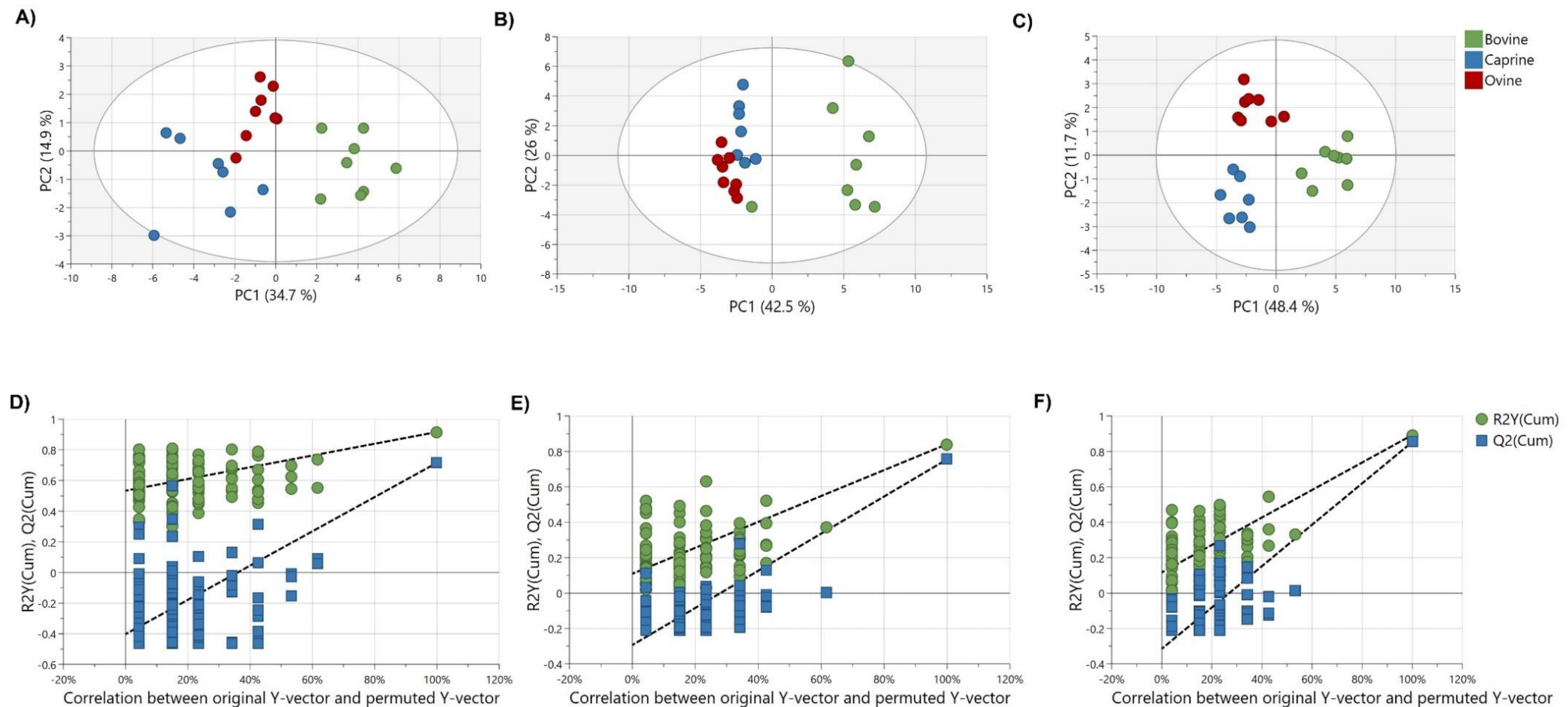


**Figure 4-3 Partial least squares discriminant analysis (PLS-DA) of lipid metabolite relative intensity differences for brain tissue samples of piglets exclusively fed whole bovine, caprine, or ovine milk treatment for 15 days.**

Score plot of A) hippocampus, B) prefrontal cortex, C) and striatum. Permutation plot of D) hippocampal, E) prefrontal cortex, and F) striatal tissue. Permutation plots involve 100 permutation tests showing no overfitting of the PLS-DA model, confirming the robustness of the model. The criteria for evaluating whether there is overfitting of the PLS-DA model is that Q2 and R2 values of the permutated Y model to the left were lower than the Q2 and R2 value of the original

model to the far right. The first two principal components (PC) are plotted. Percentages of variation explained by each PC are indicated along the axes. PC, principal component. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum

**Table 4-4 Brain lipids with a significant difference in relative intensity for brain tissue samples of piglets exclusively fed raw whole bovine, caprine, or ovine milk treatment for 15 days[+]**

| Region | Lipids | VIP | FDR | $Log_2$fold change | | |
|---|---|---|---|---|---|---|
| | | | | Ovine vs Bovine | Ovine vs Caprine | Caprine vs Bovine |
| Hippocampus | DG 41:6 | 2.5 | <0.01 | -0.14 | 0.14 | -0.29 |
| | PS 36:1 | 2.26 | 0.02 | -0.71 | 2.9 | -3.60* |
| | PS 40:5 | 2.74 | <0.01 | 0.59 | -0.82 | 1.40* |
| | TG 48:0 | 1.93 | 0.03 | -0.24 | -0.04 | -0.19 |
| | TG 48:1 | 2.47 | <0.01 | -0.19 | 0.1 | -0.29 |
| Prefrontal cortex | DG 41:6 | 2.12 | <0.01 | 0.92 | 0.11 | 0.81 |
| | PC O-37:9 | 1.65 | 0.02 | 0.26 | -0.02 | 0.28 |
| | PS 36:2 | 1.77 | 0.02 | 0.93 | 0.24 | 0.7 |
| | PS 38:4 | 2.14 | <0.01 | 1.53* | 0.06 | 1.47* |
| | PS 40:5 | 2.03 | <0.01 | 1.59* | -0.06 | 1.66* |
| Striatum | Cer 36:1;2O | 1.76 | 0.05 | -0.63 | -0.87 | 0.24 |
| | Cer 38:3;3O | 1.06 | 0.01 | -3.09* | -0.51 | -2.58 |
| | Cer 44:4;3O | 1.33 | <0.01 | -3.00* | -0.46 | -2.54* |

| | | | | | |
|---|---|---|---|---|---|
| DG 41:6 | 1.58 | 0.01 | -0.07 | 0.27 | -0.35 |
| HexCer 42:1;3O | 0.87 | 0.05 | -2.7 | -0.18 | -2.53 |
| PC 30:0 | 1.14 | 0.04 | -0.47 | 0.13 | -0.6 |
| PC 32:1 | 1.1 | <0.01 | -1.33* | -0.19 | -1.15* |
| PC 34:2 | 1.11 | <0.01 | -2.22* | -0.11 | -2.11* |
| PC 35:5 | 1.05 | 0.01 | -1.59* | -0.29 | -1.29 |
| PC 36:1 | 1.06 | <0.01 | -1.31 | -0.16 | -1.06* |
| PC 36:2 | 1.2 | <0.01 | -1.75* | -0.13 | -1.62* |
| PC 37:4 | 1.16 | <0.01 | -2.05* | -0.21 | -1.84* |
| PC 38:4 | 1.21 | <0.01 | -1.77* | -0.37 | -1.41* |
| PC 38:6 | 1.13 | 0.05 | -0.53 | -0.23 | -0.31 |
| PC 40:6 | 1.2 | <0.01 | -1.89* | -0.16 | -1.74* |
| PC 40:7 | 1.13 | <0.01 | -0.75 | -0.14 | -0.61 |
| PC 42:10 | 1.15 | <0.01 | -0.5 | -0.08 | -0.42 |
| PC O-30:0 | 1.18 | <0.01 | -2.51* | 0.62 | -3.13* |
| PC O-34:0 | 1.06 | <0.01 | -2.74* | -0.2 | -2.54* |
| PC O-37:8 | 1.2 | <0.01 | -1.59* | -0.26 | -1.34* |
| PC O-37:9 | 1.4 | <0.01 | 0.42 | 0.04 | 0.38 |

| | | | | | |
|---|---|---|---|---|---|
| PC O-39:10 | 1.4 | 0.03 | 0.18 | 0.06 | 0.12 |
| PE 34:2 | 1.19 | <0.01 | -4.47* | -0.37 | -4.10* |
| PE 36:4 | 1.17 | <0.01 | -5.19* | -0.21 | -4.98* |
| PE 40:6 | 1.16 | <0.01 | -2.85* | -0.14 | -2.71* |
| PE P-34:2 | 1.09 | <0.01 | -5.12* | -0.55 | -4.58* |
| PE P-36:4 | 1.16 | 0.04 | -5.19 | -0.3 | -0.48 |
| PE P-38:5 | 1.11 | <0.01 | -3.36 | -0.25 | -3.11* |
| PS 36:1 | 1.15 | <0.01 | -2.44* | 0.55 | -2.99* |
| PS 38:4 | 2.24 | <0.01 | -0.45 | 1.05* | -1.50* |
| PS 40:4 | 1.29 | 0.01 | -0.74 | -0.43 | -0.3 |
| PS 40:5 | 1.36 | <0.01 | 0.41 | -0.3 | 0.72 |
| SM 36:0;2O | 1.06 | <0.01 | -3.25* | -0.02 | -3.24* |
| SM 36:0;3O | 1.3 | 0.01 | -2.43* | -0.35 | -2.08* |
| SM 39:7;2O | 1.27 | <0.01 | -1.05* | 0.05 | -1.10* |
| TG 48:0 | 1.23 | <0.01 | -0.75 | 0.11 | -0.86 |
| TG 48:1 | 1.33 | <0.01 | -0.79 | 0.23 | -1.02 |
| TG 50:0 | 1.16 | <0.01 | -1.35* | 0.09 | -1.44* |
| TG 50:1 | 1.25 | <0.01 | -1.10* | 0.07 | -1.18* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | TG 50:2 | 1.35 | <0.01 | -1.04* | -0.03 | -1.01* |
| | TG 52:1 | 1.13 | <0.01 | -1.37* | 0.19 | -1.56* |
| | TG 52:2 | 1.28 | <0.01 | -1.13* | 0.19 | -1.32* |
| | TG 52:3 | 1.19 | <0.01 | -0.86 | 0.24 | -1.09* |
| | TG 54:3 | 1.31 | <0.01 | -0.5 | -0.09 | -0.42 |

*[+]Variable importance in projection (VIP) >1 from the partial least squares discriminant analysis model was used to select the contributing lipids. One-way ANOVA was used to determine the significant lipids between milk treatments (FDR < 0.05). Only lipids that satisfied multivariate and ANOVA analysis criteria are shown. *Lipids with significant $log_2$fold change > 1 or $log_2$fold change < -1 (false discovery rate (FDR) < 0.05, using t-test) between milk treatments are shown. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum; DG, diglycerides; PS, phosphatidylserine; TG, triglycerides; Cer, ceramides; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.*

#### 4.3.2.3 Correlation between milk lipid intakes and brain lipid levels

As milk treatments influenced brain lipid metabolites, a correlation analysis was performed, regardless of milk treatments, to identify whether milk lipid intakes (Appendix Table A3) influenced brain lipid relative intensities.

Significant relationships between milk lipid intakes and brain lipid relative intensities were observed (Figure 4-4 and Appendix Figure A5). However, no correlation was observed between milk intakes and brain-relative intensities of the same lipid types. For instance, striatum TG 50:0 relative intensity was not correlated to TG 50:0 in the milk (Figure 4-4).

m_TG.8.0_12.0_14.0
m_TG.9.0_9.0_16.0
m_TG.10.0_14.0_16.0
m_TG.12.0_12.0_16.0
m_TG.12.0_14.0_16.0
m_TG.13.0_15.0_19.1
m_TG.14.0_14.0_14.0
m_TG.14.0_16.0_18.2
m_TG.14.0_18.1_18.1
m_TG.16.0_16.0_16.2
m_TG.16.0_16.0_18.2
m_TG.16.0_16.0_20.3
m_TG.16.0_18.1_18.2
m_TG.26.0
m_TG.28.0
m_TG.30.0
m_TG.32.0
m_TG.32.1
m_TG.33.0
m_TG.35.0
m_TG.36.0
m_TG.36.1
m_TG.37.0
m_TG.37.1
m_TG.38.0
m_TG.38.1
m_TG.38.2
m_TG.38.3
m_TG.39.0
m_TG.39.1
m_TG.39.2
m_TG.40.1
m_TG.41.0
m_TG.41.1
m_TG.41.2
m_TG.42.1
m_TG.42.2
m_TG.42.3
m_TG.43.0
m_TG.43.1
m_TG.43.2
m_TG.44.0
m_TG.44.1
m_TG.44.2
m_TG.44.3
m_TG.45.0
m_TG.45.1
m_TG.46.0
m_TG.46.1
m_TG.46.2
m_TG.46.3
m_TG.47.0
m_TG.48.0
m_TG.48.1
m_TG.48.3
m_TG.49.0
m_TG.49.1
m_TG.49.2
m_TG.49.3
m_TG.50.0
m_TG.50.1
m_TG.50.3
m_TG.50.4
m_TG.51.0
m_TG.51.1
m_TG.51.2
m_TG.51.3
m_TG.52.1
m_TG.52.2
m_TG.52.4
m_TG.52.5
m_TG.53.2
m_TG.53.3
m_TG.53.4
m_TG.54.0
m_TG.54.1
m_TG.54.2
m_TG.54.3
m_TG.54.4
m_TG.54.5
m_TG.56.6

Cer.36.1.2O
Cer.38.3.3O
Cer.44.4.3O
DG.41.6
HexCer.42.1.3O
PC.30.0
PC.32.1
PC.34.2
PC.35.5
PC.36.1
PC.36.2
PC.37.4
PC.38.4
PC.38.6
PC.40.6
PC.40.7
PC.42.10
PC.O.30.0
PC.O.34.0
PC.O.37.8
PC.O.37.9
PC.O.39.10
PE.34.2
PE.36.4
PE.40.6
PE.P.34.2
PE.P.36.4
PE.P.38.5
PS.36.1
PS.38.4
PS.40.4
PS.40.5
SM.36.0.2O
SM.36.0.3O
SM.39.7.2O
TG.48.0
TG.48.1
TG.50.0
TG.50.1
TG.50.2
TG.52.1
TG.52.2
TG.52.3
TG.54.3

-1
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1

**Figure 4-4 Correlation plot showing Spearman correlations between milk lipid intakes (in %) and striatal lipid relative intensities in (n = 23) tissue samples obtained from piglets fed with milk from bovine, ovine or caprine species.**

The colour of the ellipse indicates the type of correlation, i.e., blue indicates a positive correlation, and red indicates a negative correlation. The ellipse shape indicates the magnitude of the correlation, i.e., the stronger the correlation, the flatter the ellipse. Only significant correlations ($P < 0.05$) are shown. The legend at the bottom of each correlation plot shows the correlation coefficients with their corresponding colours. The horizontal axis represents milk lipid intakes (preceded with the letter m), and the vertical axis represents striatal lipid relative intensities.

### 4.3.3 Lipid pathway analysis

The ovine or caprine milk groups showed a more active pathway for PE biosynthesis from PS in the hippocampus compared to the bovine milk group (Figure 4-5A-B). Conversely, the caprine milk group showed a suppressed pathway for biosynthesis of PS from PC and PC from PE compared to the bovine milk group (Figure 4-5B). Comparisons between ovine and caprine milk groups for the hippocampus did not identify any differentially activated or suppressed pathways (Appendix Figure A6A).

In the prefrontal cortex, the ovine milk group showed an active pathway for the biosynthesis of PC from PE and PE from PS and a suppressed pathway for the biosynthesis of PS from PC compared to the bovine milk group (Figure 4-5C). The ovine milk group showed an active pathway for biosynthesis of PC from PE, whereas suppressed pathway for biosynthesis of PS from PC, PE from DG, and PC from DG compared to the caprine milk group (Figure 4-5D). The caprine milk group showed an active pathway for biosynthesis of PC and PE from DG compared to the bovine milk group (Figure 4-5E).

In the striatum, the ovine milk group showed a suppressed pathway for biosynthesis of PC from DG and PS from PC compared to the bovine milk group (Figure 4-5F). The caprine milk group showed an active pathway for biosynthesis of PC from DG (DG-PE-PC), whereas suppressed pathway for biosynthesis of PC from DG compared to the bovine milk group (Figure 4-5G). No lipid pathways in the striatum were differentially activated between ovine and caprine milk groups (Appendix Figure A6B).

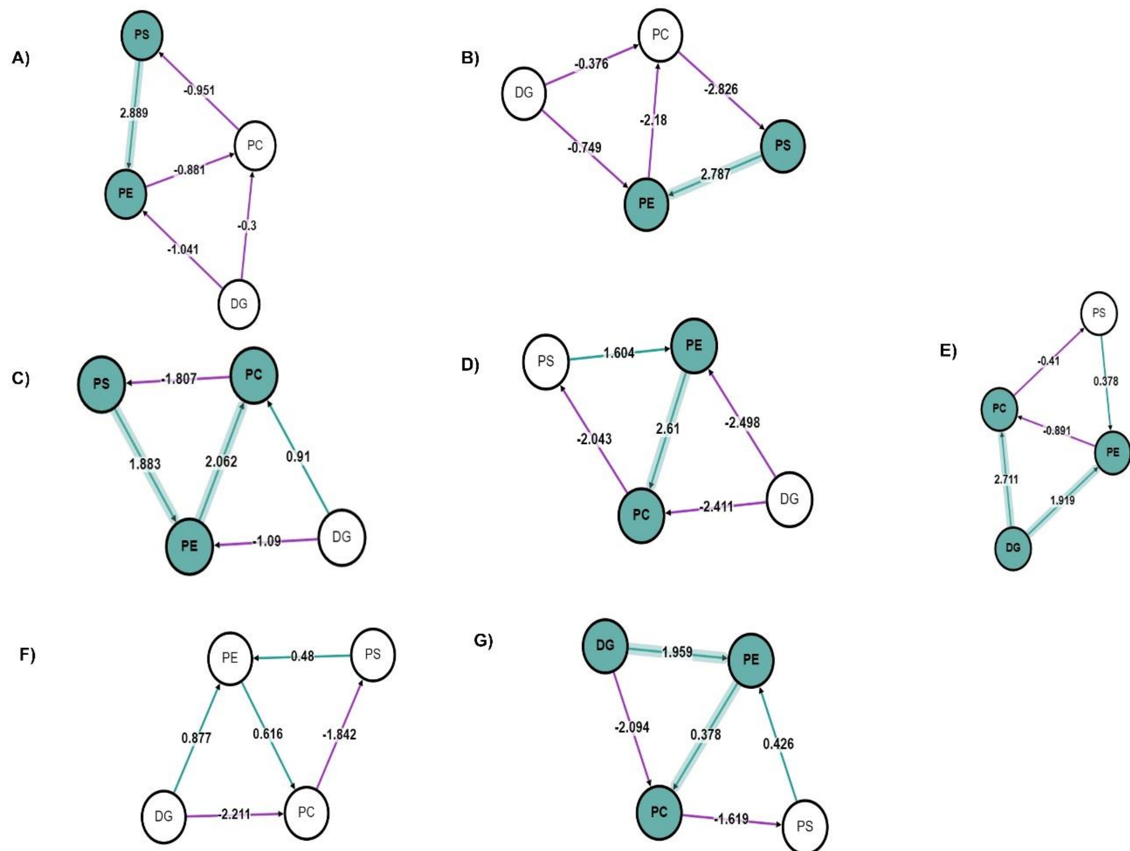


**Figure 4-5 BioPAN lipid networks.**

Lipid metabolite network exported from BioPAN in the A) hippocampus of ovine vs bovine milk groups and B) caprine vs bovine milk groups, C) prefrontal cortex of ovine vs bovine milk groups, D) ovine vs caprine milk groups, and E) caprine vs bovine milk groups; F) striatum ovine vs bovine milk groups and G) caprine vs bovine milk groups. Green nodes correspond to active lipids, and green shaded arrows to active pathways. Reactions with positive Z scores have green arrows, while reactions with negative Z scores are coloured purple. Only the pathways with a P-value < 0.05 were considered significant (Z-score equivalent > 1.645).

## 4.4 Discussion

This study is the first to provide evidence of the effects of exclusively feeding whole bovine, caprine, or ovine milk on the metabolome of brain areas associated with cognitive function, the hippocampus, prefrontal cortex, and striatum in three-week-old piglets as a model for human infants. The results showed that the relative intensities of brain polar and non-polar (lipid) metabolites change in response to different milk treatments. In addition, the relative intensities of striatal lipid metabolites were most affected by milk treatments, suggesting a brain region-specific milk effect. Another finding was that metabolites differ in their relative intensities between brain regions, regardless of the milk treatments.

### 4.4.1 Metabolites differences between brain regions

Among the notable differences in brain metabolite profiles, regardless of the milk treatments, were region-specific amino acids, amino acid derivatives, phospholipids, sphingolipids and glycerolipids. These patterns are likely due to different energy requirements (Grachev and Apkarian, 2001; Watts et al., 2018) and tissue composition (Zhang and Shen, 2015) of these regions. Among polar metabolites, glutamine was present at a higher relative intensity in the prefrontal cortex than in other brain regions. Glutamine is used to synthesise glutamate and GABA neurotransmitters (Bak et al., 2006), and more glutamine in the prefrontal cortex might support higher neuronal activity involving glutamate and GABA.

Among the lipids, sphingolipids were present at higher relative intensities in the striatum than hippocampus and prefrontal cortex, likely reflecting its higher glia-to-neuron ratio in the striatum (Hofmann et al., 2017; Fitzner et al., 2020). More glia populations would

produce more sphingolipids that would ultimately be required for myelin formation (Fitzner et al., 2020).

### 4.4.2 Metabolites differences between milk treatments

Of the brain regions analysed in this study, striatal lipid profiles were most affected by ruminant milk treatments. The reason behind this preferential effect of milk on striatal lipid metabolites remains unknown. However, a study has shown a correlation between breastfeeding and ventral and dorsal striatum volume (Koshiyama et al., 2020). Hence, it could be speculated that the striatum might be more malleable to dietary interventions than the prefrontal cortex and hippocampus.

Irrespective of the brain region, the intensity of glycerophospholipids (PS, PC, PE), glycerolipids (TG, DG), and sphingolipids (CER, SM) differed between milk treatments. Similarly, a study in piglets has shown that hippocampus TG levels were increased in response to infant formula supplemented with a high level (8 %) of MFGM compared to the control unsupplemented infant formula (Fraser et al., 2022). Other rodent studies have shown that phospholipids serve as a precursor for acetylcholine (Jope and Jenden, 1979; Lopez et al., 1991). Considering the role of acetylcholine in learning and memory (Haam and Yakel, 2017; Maurer and Williams, 2017), it is likely that a diet enriched with phospholipids would increase the substrate for acetylcholine synthesis, thereby influencing cognitive functioning. In addition, studies showed an association between increased blood TG levels and decreased brain executive functions and memory in healthy older adults (De Frias et al., 2007; Parthasarathy et al., 2017). However, whether changes in these dietary and blood lipids alter the brain lipids profile, thereby influencing brain development, remains to be explored.

Notable differences in the relative intensity of brain lipid metabolites were only observed between bovine and other milk (caprine or ovine) groups. This observation could be ascribed to differences in the milk lipid intakes between bovine, caprine or ovine milk (Appendix TableA3). However, there were no correlations between intakes and relative intensities of the same lipid species in different brain regions. The complex digestion and absorption of lipids could explain this lack of correlation. Hydrolysis of ingested lipids occurs in the small intestinal lumen, resulting in fatty acids and monoglycerides that enter the enterocytes for re-esterification and subsequent packing into chylomicrons, which are transported via the lymph to the bloodstream and then to body tissues. Hence, continuous breakdown and reassembly of the lipid types within the enterocytes might have resulted in new lipid species that are different to that of the ingested milk lipids.

Additionally, predicted affected lipid pathways might partially explain some correlations between milk lipid intakes and brain lipid metabolite relative intensities. For instance, in this study, some of the milk TG species were positively and negatively correlated with PC species in the striatum, and interestingly, TG can be metabolised to DG (Alves-Bezerra and Cohen, 2018). Furthermore, lipid pathway analysis showed that DG serves as a precursor of PE, producing PC, supporting the correlation between milk TG intakes and striatal PC relative intensities.

Compared to lipids, polar metabolite profiles in the brain areas were less influenced by milk treatments. The relative intensity of threonine was altered between milk treatments in the hippocampus and striatum, despite similar milk threonine intake between piglets from different milk groups (Appendix Table A4). The changes in relative intensities of threonine might alter brain neurotransmission. For example, threonine is an essential amino acid that acts as a precursor for the neurotransmitter glycine (Boehm et al., 1998), which plays an important role in memory and other neurological functions (Ullah et al.,

2020). However, whether a change in brain threonine levels between milk treatments was sufficient to change brain neurotransmission is unknown.

How lipids and amino acids in milk affect brain lipids levels remains unknown. Some studies have suggested that dietary-derived lipids may cross the BBB (Eguchi et al., 2020; Pifferi et al., 2021), mainly fatty acids (Strosznajder et al., 1996; Mitchell et al., 2011; Pan et al., 2015). They can be reassembled in the brain to produce other brain lipid species. Similarly, amino acids can cross the BBB (Zaragozá, 2020).

### 4.4.3 Lipid pathways differences between milk treatments

The BioPAN analysis of the brain lipid metabolite data suggested that pathways involving glycerophospholipid (PC, PE, PS) biosynthesis were most affected by milk treatments. Milk is a good source of glycerophospholipids (Schverer et al., 2020), and these complex lipids differ in concentrations between bovine, caprine and ovine milk (Zancada et al., 2013; Castro-Gómez et al., 2014), with ovine and caprine milk containing more phospholipids than bovine milk. In addition, glycerophospholipids play a role in neural membrane stability, permeability, fluidity and proper functioning of membrane protein, receptors, and ion channels in the brain (Farooqui et al., 2000), suggesting a high requirement for glycerophospholipids for brain functioning.

Bovine milk was predicted to have the most effect on the glycerophospholipid biosynthesis pathways. However, the active and suppressed states of these pathways were predicted to be brain region-specific. For instance, in the bovine milk group, the activity of PE biosynthesis pathways was predicted to be suppressed in the hippocampus and prefrontal cortex, whereas its activity was predicted to increase in the striatum compared with other milk groups. The fatty acid composition of PE could explain this brain region-dependent activity. PE fatty acid composition varies between grey and white matter, with

more 16:0, 16:1, 18:0, and 18:1 fatty acids in grey than white matter (O'Brien et al., 1964; Svennerholm, 1968; Kim et al., 2014). Considering the possible lower grey-to-white matter ratio in the striatum compared to the prefrontal cortex and hippocampus (Hofmann et al., 2017; Fitzner et al., 2020), there were likely less 16:0, 16:1, 18:0, and 18:1 fatty acids in the striatum. These fatty acids are also in higher amounts in bovine milk (Vaz et al., 2011). The milk fatty acid profile was not ascertained in this study. However, it could be speculated that a higher amount of these fatty acids in the bovine milk intake (Vaz et al., 2011) might have suppressed the PE biosynthesis pathway in the hippocampus and the prefrontal cortex, as these brain areas have higher intrinsic fatty acids (including 16:0, 16:1, 18:0, and 18:1) and they may not require more of the fatty acids coming from the diet for PE synthesis.

### 4.4.4 Strength and limitations

The main strength of this study was the use of metabolomics for profiling the brain metabolites, which allowed the detection of many diverse metabolites compared to quantitative methods, which only detect specific metabolites. However, there are some limitations. The study presented a brain region-specific metabolite profile. However, there might be potential issues related to the manual dissection of visually challenging brain areas, which could lead to the dilution of regional differences due to milk treatments. Additionally, this study does not show the physiological consequences of changes in the brain metabolome profile in response to milk treatments. Limitations of the LC-MS-based metabolomics approach are that it provides relative quantification of metabolites, but not absolute quantification and many features remain unidentified.

Furthermore, only brain metabolite intensities were measured in male piglets. A study on adult rats showed that brain metabolites were sex-dependent, with glutamate, creatine, and myoinositol levels higher in male rats than female rats (Mansouri et al., 2012). Hence,

including female piglets in the study would have been informative, but would require more piglets per treatment.

## 4.5 Conclusion

This study provides the first evidence of associations of exclusively feeding whole bovine, caprine, or ovine milk with changes in metabolites of brain areas associated with cognitive function, hippocampus, prefrontal cortex, and striatum of three-week-old piglets in the early postnatal period. LCMS-metabolomics analysis showed that the relative intensities of glycerophospholipids, glycerolipids, sphingolipids, and threonine were altered in the brain tissues of piglets fed the milk treatments. The analysis of the brain metabolome also highlighted the difference in profile across brain regions, predominantly the striatal profile, pointing to the heterogeneous development. It remains to be elucidated whether the brain region-specific milk effects observed here would be sufficient to change the molecular and physiological features associated with the cognitive development of the piglets at ~PND21 and longer term.

# Chapter 5

# Gene expression profiling of cognitive areas of the brain in response to ruminants' milk in early postnatal life

The contents of this chapter have been published as a peer-reviewed paper: **Jena A**, Montoya CA, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC and Roy NC (2022) The effects of ruminant milk treatments on hippocampal, striatal, and prefrontal cortex gene expression in pigs as a model for the human infant. Frontiers in Neuroscience.16:937845. doi: 10.3389/fnins.2022.937845

## Abstract

While infant formula is usually bovine milk-based, interest in other ruminant milk-based formula is growing. However, whether different ruminant milk treatments influence the underlying molecular features of the developing brain differently remains unknown. Therefore, the aim was to determine the effects of consuming bovine, caprine, or ovine milk on brain gene expression in the early postnatal period using a piglet model of the human infant. Piglets were exclusively fed raw whole bovine, ovine, or caprine milk starting at PND 7 or 8 for 15 days. The mRNA abundance of 77 genes in the prefrontal cortex, hippocampal, and striatal regions was measured at PND 21 or 22 using NanoString. The expression level of two hippocampal and nine striatal genes was affected by milk treatments, particularly ovine milk. These modulatory genes are involved in glutamate, GABA, 5-HT, adrenaline and neurotrophin signalling and the synaptic vesicle cycle. In addition, the expression level of genes involved in GABA signalling was correlated with piglets' lactose intake. In contrast, milk treatments did not affect gene expression in the prefrontal cortex. This study provides the first evidence of the association of different ruminant milk treatments with brain gene expression related to cognitive function in the first three months of postnatal life.

## 5.1 Introduction

The early postnatal years of life are critical in determining developmental, behavioural, and health outcomes in later life. Rapid synaptogenesis, myelination, and the establishment of cognitive abilities mark developmental changes in this period. In addition, genetic (e.g., brain-derived neurotrophic factor (BDNF), neuregulin) and environmental (e.g., nutrition, prenatal care) factors influence the development of the brain (Valadares et al., 2010; Knickmeyer et al., 2014). Genetic factors dictate the *in utero* brain development stages, but environmental factors mainly influence postnatal brain development. Hence, any environmental insult or stimuli during this period could affect brain performance in adulthood.

Human breast milk is the optimal source of nutrition for infants, but infant formula is an alternative or complementary solution when human breast milk is unavailable or limited. Milk, regardless of the species it comes from, contains different lipids (e.g., phospholipids, SM, PUFA), proteins (e.g., lactoferrin), carbohydrates (e.g., lactose, oligosaccharides), vitamins and minerals (e.g., vitamin B, choline, iron), with many of these nutrient sources increasingly recognised for their associations or roles in brain development postnatally (Lin et al., 2019).

A study by Deoni et al., 2018 showed that a formula containing a high amount (62 mg/L) of SM increases brain myelination and improves cognitive performance in infants compared with a low amount (28 mg/L) of SM. Another study reported an association between the concentration of oligosaccharide 2-fucosyllactose in breast milk at one month of lactation and improved cognitive function in infants aged 24 months (Berger et al., 2020). In addition, inadequate protein intake during early postnatal life was associated

with learning and memory impairments, reduced brain weight, and dendritic arborisations in rodents (Valadares et al., 2010; Chertoff, 2015).

The effects of early postnatal nutrition on brain development have been studied primarily in the context of behaviour, but fewer studies have focused on the underlying changes in molecular features in the brain tissue (Fleming et al., 2020; Page and Anday, 2020). For instance, a study showed that feeding HMO or bovine milk oligosaccharides from PND 2 to 32 to piglets either increases or decreases the hippocampal expression of neurotransmitter receptor (*GABRB2*, *GLRA4*, *CHRM3*) and transporter (*SLC1A7*) genes and improves recognition memory, suggesting a link between nutrient-gene-behaviour (Fleming et al., 2020). Hence, more studies are essential to better understand the effect of nutrition on brain gene expression that may act as a determinant of cognitive abilities and behaviour in early postnatal life.

Bovine milk is the most common ruminant milk used for infant formula (Martin et al., 2016). However, caregivers have increased interest in using formula made with other ruminant milk, including ovine and caprine sources. This expansion in the use of non-bovine milk is primarily due to the association of bovine milk consumption with the development of allergies in infants and reduced symptoms with consuming ovine or caprine milk (Park, 1994; Masoodi and Shafi, 2010). In addition, ovine and caprine milk have a greater nutritional value than bovine milk for specific nutrients. For example, ovine milk contains higher proteins, lipids, vitamins (riboflavin and vitamin C), minerals (calcium and phosphorous), and energy (Park et al., 2007; Barlowska et al., 2011; Claeys et al., 2014), while caprine milk has more oligosaccharides and a profile of oligosaccharides closer to that of human breast milk (Viverge et al., 1997; Martinez-Ferez et al., 2006).

Previous *in vitro* (Roy et al., 2021) and *in vivo* (Roy et al., 2022a) studies have shown that gastric digestion and stomach emptying rate of nutrients differed across ruminant milk. These differences would likely affect the availability of nutrients for small intestinal absorption, fermentation by the resident microbiota in the gut, tissue metabolism, and brain function. Another *in vitro* study showed that pre-digested bovine and ovine milk fermentation using infant faecal inoculum resulted in differences in the relative abundance of the microbiota and their metabolites between milk types (Ahlborn et al., 2020). In addition, microbiota and microbial metabolites are increasingly recognised for their potential to influence brain function by participating in the GBA (O'Mahony et al., 2015; Gao et al., 2019; Kelsey et al., 2021; Tamana et al., 2021). However, despite these differences between ruminant milk, their effects on the activity of the different brain regions with specific roles in cognitive development (e.g., the hippocampus, striatum, and prefrontal cortex) (Lavenex and Banta Lavenex, 2013; Wierenga et al., 2014; Werchan et al., 2016), are ill-defined.

Based on the published evidence, it was hypothesised that differences in nutrient composition between the three main ruminant milk used to make infant formula would lead to differences in gene expression of the brain areas associated with cognitive function. Therefore, the aim was to determine the effects of bovine, ovine, and caprine milk on the gene expression of the prefrontal cortex, hippocampus, and striatum in piglets as a model of human infants. In addition, the gene expression profile of these brain areas was compared, regardless of the milk treatments.

## 5.2 Materials and methods

### 5.2.1 Milk chemical composition analysis

The dry matter content of milk was analysed using an air oven-drying method 990.19, milk proteins using the Dumas method 968.06, and milk fats using the Mojonnier method 989.05, respectively. In addition, milk lactose content was measured using a spectrophotometric enzymatic kit (catalogue no.-10176303035) (R-Biopharm AG, Germany), and gross energy content was measured using a LECO AC500 bomb calorimeter (LECO Corporation, USA).

### 5.2.2 Animal study

The study design was described in Chapter 3 (c.f., section3.2.2).

### 5.2.3 Brain tissue sampling

The procedure for sampling was described in Chapter 4 (c.f., section 4.2.2).

### 5.2.4 RNA extraction

Each brain tissue sample (10 to 20 mg) was homogenised using a handheld homogeniser for 60 sec in 1 mL of QIAzol lysis reagent (Qiagen, Germany). The total RNA was extracted using an RNeasy lipid tissue mini kit following the manufacturer's instructions (Qiagen, Germany) and dissolved in 50 µL of RNAase-free water. The concentration and the quality of the extracted RNA were evaluated using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and the Agilent 2100 Bioanalyser (Agilent Technologies, USA), respectively. Samples with an RNA integrity number greater than six were used for gene expression analysis, and this criterion was satisfied by all the brain tissue samples.

### 5.2.5 Gene panel selection

A customised panel of 150 genes was created using a literature search and pre-existing NanoString human gene panel for learning, memory, and neurotransmission. Then, the curation of genes was carried out based on protein existence and entry status in Uniprot. First, the genes whose evidence was available at transcript and protein levels and whose entry status was reviewed were selected, making a panel of 77 genes (Table 5-1). The panel consisted of genes related to the cellular process in the brain (synaptogenesis, myelination, neurotrophins, and synaptic vesicle cycle) and neurotransmission (neurotransmitter receptor, transporter, and enzymes involved in neurotransmitter synthesis).

**Table 5-1 Full NanoString list of genes associated with brain cellular processes and neurotransmission**

| Gene symbol | Gene name |
|---|---|
| *ACHE* | Acetylcholinesterase |
| *ADRA1D* | Adrenoceptor Alpha 1D |
| *ADRA2A* | Adrenoceptor Alpha 2A |
| *ADRA2B* | Adrenoceptor Alpha 2B |
| *ADRB1* | Adrenoceptor Beta 1 |
| *ADRB2* | Adrenoceptor Beta 2 |
| *ADRB3* | Adrenoceptor Beta 3 |
| *AHR* | Aryl hydrocarbon receptor |
| *BDNF* | Brain-derived neurotrophic factor |
| *CHRM1* | Cholinergic Receptor Muscarinic 1 |
| *CHRM2* | Cholinergic Receptor Muscarinic 2 |
| *CHRM3* | Cholinergic Receptor Muscarinic 3 |
| *CHRNA7* | Cholinergic Receptor Nicotinic Alpha 7 Subunit) |
| *CHRNB2* | Cholinergic Receptor Nicotinic Beta 2 Subunit |
| *CNP* | 2',3'-Cyclic Nucleotide 3' Phosphodiesterase |
| *CPLX1* | Complexin 1 |
| *CPLX3* | Complexin 3 |
| *CPLX4* | Complexin 4 |
| *DBH* | Dopamine Beta-Hydroxylase |
| *DDC* | Dopa Decarboxylase |
| *DLG4* | Discs Large MAGUK Scaffold Protein 4 |
| *DRD1* | Dopamine Receptor D1 |
| *DRD2* | Dopamine Receptor D2 |
| *GABBR1* | Gamma-Aminobutyric Acid Type B Receptor Subunit 1 |
| *GABRA1* | Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha1 |
| *GABRB2* | Gamma-Aminobutyric Acid Type A Receptor Subunit Beta2 |
| *GABRB3* | Gamma-Aminobutyric Acid Type A Receptor Subunit Beta3 |
| *GABRG2* | Gamma-Aminobutyric Acid Type A Receptor Subunit Gamma2 |

| | |
|---|---|
| *GAD1* | Glutamate Decarboxylase 1 |
| *GAD2* | Glutamate Decarboxylase 2 |
| *GAP43* | Growth Associated Protein 43 |
| *GLS* | Glutaminase |
| *GRIA2* | Glutamate Ionotropic Receptor AMPA Type Subunit 2 |
| *GRID1* | Glutamate Ionotropic Receptor Delta Type Subunit 1 |
| *GRID2* | Glutamate Ionotropic Receptor Delta Type Subunit 2 |
| *GRM1* | Glutamate Metabotropic Receptor 1 |
| *GRM2* | Glutamate Metabotropic Receptor 2 |
| *GRM6* | Glutamate Metabotropic Receptor 6 |
| *GRM7* | Glutamate Metabotropic Receptor 7 |
| *GRM8* | Glutamate Metabotropic Receptor 8 |
| *HRH1* | Histamine Receptor H1 |
| *HTR1B* | 5-Hydroxytryptamine Receptor 1B |
| *HTR1D* | 5-Hydroxytryptamine Receptor 1D |
| *HTR1E* | 5-Hydroxytryptamine Receptor 1E |
| *HTR2A* | 5-Hydroxytryptamine Receptor 2A |
| *HTR2B* | 5-Hydroxytryptamine Receptor 2B |
| *HTR2C* | 5-Hydroxytryptamine Receptor 2C |
| *HTR4* | 5-Hydroxytryptamine Receptor 4 |
| *MAG* | Myelin Associated Glycoprotein |
| *MBP* | Myelin Basic Protein |
| *NPY1R* | Neuropeptide Y Receptor Y1 |
| *NPY2R* | Neuropeptide Y Receptor Y2 |
| *NPY5R* | Neuropeptide Y Receptor Y5 |
| *NTF3* | Neurotrophin 3 |
| *NTRK3* | Neurotrophic Receptor Tyrosine Kinase 3 |
| *PLP1* | Proteolipid Protein 1 |
| *RAB3A* | Ras-Related Protein Rab-3A |
| *RIMS1* | Regulating Synaptic Membrane Exocytosis 1 |

| | |
|---|---|
| *SLC1A1* | Solute Carrier Family 1 Member 1 |
| *SLC1A2* | Solute Carrier Family 1 Member 2 |
| *SLC1A3* | Solute Carrier Family 1 Member 3 |
| *SLC22A1* | Solute Carrier Family 22 Member 1 |
| *SLC22A2* | Solute Carrier Family 22 Member 2 |
| *SLC22A3* | Solute Carrier Family 22 Member 3 |
| *SLC5A7* | Solute Carrier Family 5 Member 7 |
| *SLC6A1* | Solute Carrier Family 6 Member 1 |
| *SLC6A11* | Solute Carrier Family 6 Member 11 |
| *SLC6A13* | Solute Carrier Family 6 Member 13 |
| *SNAP25* | Synaptosome Associated Protein 25 |
| *STX1B* | Syntaxin 1B |
| *STX3* | Syntaxin 3 |
| *STXBP1* | Syntaxin Binding Protein 1 |
| *SYN1* | Synapsin I |
| *SYN2* | Synapsin II |
| *SYN3* | Synapsin III |
| *SYT1* | Synaptotagmin 1 |
| *VAMP2* | Vesicle Associated Membrane Protein 2 |

### 5.2.6 Gene expression analysis

Seventy-seven genes were detected using the NanoString nCounter™ system (NanoString Technologies, United States). The starting amount of RNA was 300 ng. Each RNA sample of 7 µL was mixed with colour-coded capture and reporter probe provided by NanoString. The samples and probes were hybridised at 67°C for 22 h. After hybridisation, samples were run on the NanoString nCounter prep station, removing excess probes and immobilising the sample-probe complex on a cartridge. A nCounter

digital analyser counted the immobilised colour-coded complex on the surface of the cartridge.

### 5.2.7 Data processing

The raw data (reporter code count files) generated by the analyser was uploaded into nSolver software (Version 4.0, NanoString Technologies, United States). Quality control checks were performed on the raw data using nSolver's default settings. First, a background correction was carried out by subtracting the number of counts of the highest negative control (out of six negative internal controls provided by NanoString) plus two standard deviations from all the mRNA counts. This step was followed by normalising individual mRNA counts against the geometric mean of six NanoString positive internal control oligonucleotides and seven reference genes: actin-beta (*ACTB*), beta-2-microglobulin (*B2M*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), lactate dehydrogenase A (*LDHA*), phosphoglycerate kinase 1 (*PGK1*), peptidylprolyl isomerase A (*PPIA*), and ribosomal protein L4 (*RPL4*).

### 5.2.8 Statistical analyses

All statistical analyses were performed using R (version 4.02). Normalised mRNA count data were log2-transformed prior to statistical analysis. PCA was performed to compare overall gene expression profiles between treatments. One hippocampal tissue sample of the ovine milk group was identified as an outlier using the criteria of > 3 standard deviations from the mean and Hotelling T2 plot (Appendix Figure A7) and was removed from the study. Differences in expression levels of individual genes between treatment groups and brain regions were analysed by one-way ANOVA using the rstatix R package. Brain tissues are different from each other in terms of structure and function. Thus, a one-way ANOVA for each tissue was conducted to compare the effect of milk treatments. An

FDR correction was used to reduce the risk of false positives. Genes with FDR < 0.05 were considered significantly different, but when the significance between groups could not be identified with FDR < 0.05, FDR < 0.1 was considered significant. The Fisher's least significant difference test was used for posthoc analysis, performed using the agricolae package for R. Volcano plots were used to visualise the significant pairwise differential expression of genes between milk treatment with log2FC on the X axis and -log2(adjusted P value) along the Y axis. Genes with log2 FC > 0.5849 or log2 FC < -0.5849 (equivalent to FC >1.5) and FDR < 0.05 (identified using a t-test) were considered differentially expressed. Differential gene expression between brain regions was visualised using the pheatmap R package.

The association between nutrient intake (average daily intake) and brain gene expression was assessed irrespective of the milk treatments, using Spearman correlations. Milk nutrient intake was calculated from PND 9 or 10 onwards (Appendix Table A5), as the first three days of the acclimatisation phase had a substantial amount of milk spills and refusals. Spearman rank correlation coefficient and the corresponding P-value were calculated using the cor.test function and visualised using the corrplot package in R. Correlations with P < 0.05 and rho > 0.5 or rho < -0.5 were considered significant.

## 5.3 Results

### 5.3.1 Overview of gene expression

Gene expression patterns differed between the brain regions (Figure 5-1). The largest variation in gene expression profiles of brain tissue samples was seen in the principal component 1, with samples primarily grouped by brain region. Gene expression profiles in the striatum were distinct from the other areas, whereas the hippocampal and prefrontal cortex profiles overlapped to some extent. A secondary grouping of samples based on

milk treatments was observed for the striatum for caprine and ovine milk-fed piglets, but this grouping was less evident for the hippocampus and prefrontal cortex.

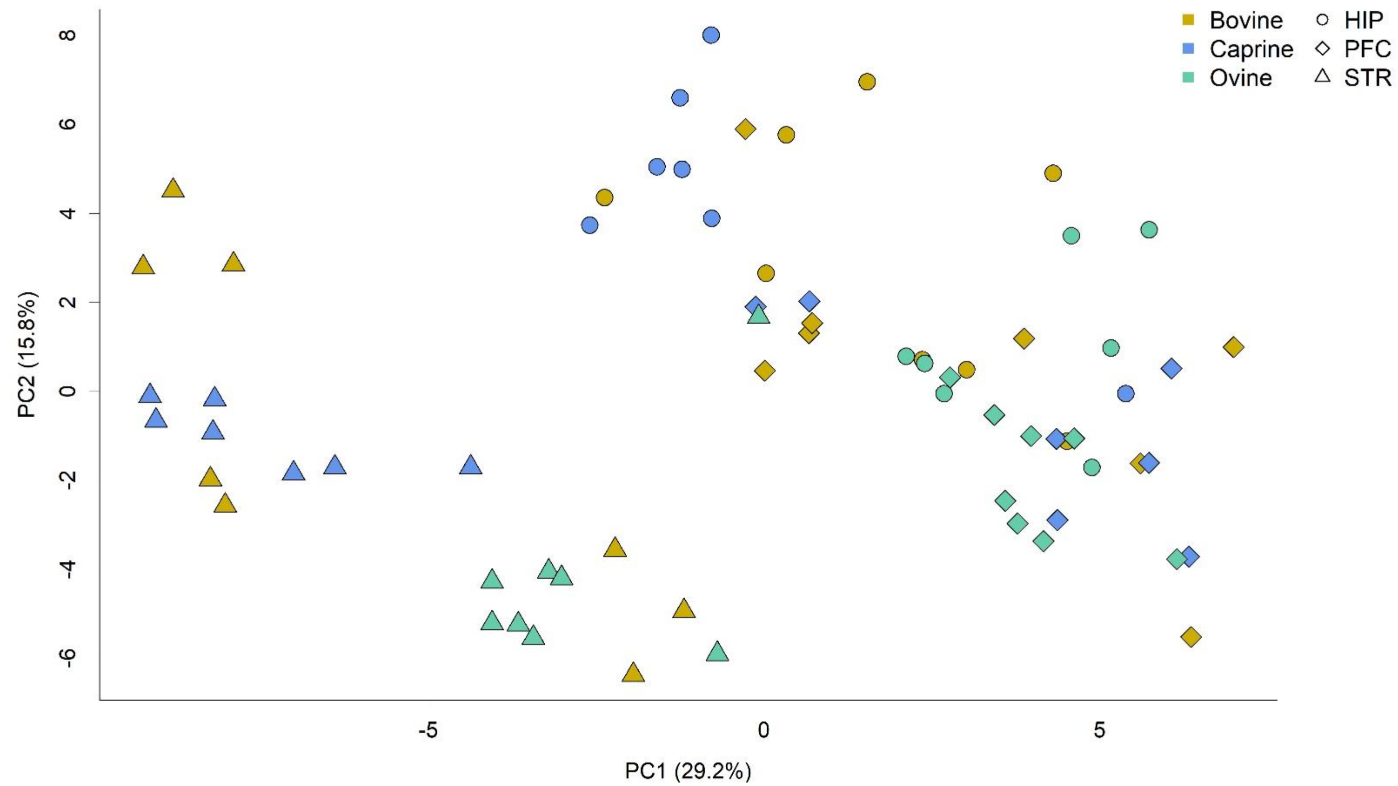


**Figure 5-1 Principal component analysis score plot showing gene expression profiles of tissue samples from the hippocampus, striatum and prefrontal cortex of piglets fed milk from bovine, caprine or ovine species.**

The first two principal components (PC) are plotted. Colours indicate milk treatments, and shape indicates different brain regions. Percentages of variation explained by each principal component are indicated along the axes. HIP, hippocampus; PFC, prefrontal cortex; STR, striatum; PC, principal component.

## 5.3.2 . Differential gene expression

### 5.3.2.1 Brain region effect

Differences in gene expression levels between brain regions were observed, with 54 out of 77 genes being significantly different (FDR < 0.05) (Table 5-2). Genes encoding for receptors for neurotransmitter glutamate (*GRID1*, *GRID2*, *GRM1*, *GRM7, GRM8* except for *GRM2*), 5-HT (*HTR1B*, *HTR1D*, *HTR2C*, *HTR4* except for *HTR1E*), dopamine (*DRD1* and *DRD2*), neuropeptide Y (*NPY1R, NPY5R* except for *NPY2R*), and norepinephrine (*ADRA2B*, *ADRB1*, *ADRB2* except for *ADRA1D*, and *ADRA2A*) had in general higher expression levels in the striatum than that of hippocampus and prefrontal cortex. Conversely, lower expression levels of genes encoding for receptors for neurotransmitter *GABA* (*GABRB2*, *GABRG2*, *GABRA1* except for *GABBR1*), histamine (*HRH1*), and acetylcholine (*CHRM3*) were observed in the striatum in comparison to the prefrontal cortex or hippocampus (Table 5-2).

The genes encoding for enzymes involved in the synthesis of the neurotransmitter acetylcholine (*ACHE*), dopamine (*DDC*), glutamate (*GAD1* and *GAD2*), norepinephrine (*DBH*), and transporters of neurotransmitter glutamate (*SLC1A2* and *SLC1A3*), acetylcholine (*SLC5A7*), and GABA (*SLC6A1* and *SLC6A11*) had in general higher expression levels in the striatum than those of the hippocampus and prefrontal cortex. In addition, myelination marker (*MAG*, *MBP*, *PLP1*, and *CNP*) gene expression levels were higher in the striatum and hippocampus compared to the prefrontal cortex.

**Table 5-2 Expression levels of genes in the hippocampus (n = 22), prefrontal cortex (n = 23), and striatum (n = 23) tissue samples of piglets fed ovine, bovine, or caprine milk treatment*.**

| Category | Gene | Hippocampus | Prefrontal cortex | Striatum | FDR |
|---|---|---|---|---|---|
| Myelination marker | *CNP* | 1305.22 ± 20.22[a] | 1156.28 ± 18.42[b] | 1272.62 ± 12.5[a] | 2.63E-07 |
| | *MAG* | 1025.21 ± 21.66[a] | 848.15 ± 24.28[b] | 996.89 ± 16.09[a] | 2.51E-07 |
| | *MBP* | 1426.16 ± 24.27[a] | 1277.15 ± 24.31[b] | 1375.92 ± 17.6[a] | 9.44E-05 |
| | *PLP1* | 1360.7 ± 21.86[a] | 1198.35 ± 17.97[b] | 1326.39 ± 16.3[a] | 2.38E-07 |
| Neurotransmitter receptor | *ADRA1D* | 397.24 ± 30.22[b] | 719.69 ± 14.37[a] | 312.71 ± 38.72[c] | 1.01E-13 |
| | *ADRA2A* | 697.85 ± 21.56[a] | 683.56 ± 9.71[a] | 510.84 ± 23.64[b] | 2.54E-09 |
| | *ADRA2B* | 471.87 ± 29.64[a] | 305.36 ± 33.56[b] | 544.93 ± 16.9[a] | 3.44E-07 |
| | *ADRB1* | 633.38 ± 25.05[c] | 693.28 ± 8.89[b] | 791.14 ± 14.76[a] | 2.02E-07 |
| | *ADRB2* | 521.43 ± 11.77[ab] | 489.43 ± 22.71[b] | 557.61 ± 11.89[a] | 0.023704 |
| | *CHRM3* | 753.79 ± 8.06[c] | 909.5 ± 5.73[a] | 803.39 ± 12.45[b] | 1.34E-16 |
| | *DRD1* | 677.83 ± 19.67[c] | 816.52 ± 8.71[b] | 1198.9 ± 16.27[a] | 6.66E-32 |
| | *DRD2* | 461.92 ± 34.16[b] | 374.39 ± 25.21[b] | 1203.17 ± 34.25[a] | 1.26E-27 |
| | *GABBR1* | 1264.34 ± 7.55[b] | 1270.26 ± 7.29[b] | 1364.38 ± 8.89[a] | 3.47E-13 |
| | *GABRA1* | 1140.18 ± 17.52[b] | 1255.49 ± 10.41[a] | 1078.95 ± 12.53[c] | 4.20E-12 |
| | *GABRB2* | 1092.72 ± 13.67[c] | 1198.45 ± 7.53[a] | 1151.92 ± 10.29[b] | 3.38E-08 |
| | *GABRG2* | 1158.47 ± 9.32[a] | 1164.38 ± 8.04[a] | 1116.36 ± 6.89[b] | 0.000204 |
| | *GRID1* | 862.76 ± 7.84[c] | 891.95 ± 4.54[b] | 986.23 ± 6.76[a] | 3.51E-19 |

| | | | | | |
|---|---|---|---|---|---|
| | *GRID2* | 498.73 ± 49.82$^{b}$ | 403.49 ± 49.75$^{b}$ | 652.71 ± 50.06$^{a}$ | 0.004225 |
| | *GRM1* | 752.41 ± 15.89$^{b}$ | 617.43 ± 27.74$^{c}$ | 811.72 ± 8.7$^{a}$ | 7.52E-09 |
| | *GRM2* | 475.43 ± 34.2$^{b}$ | 623.42 ± 21.61$^{a}$ | 476.42 ± 31.25$^{b}$ | 0.000903 |
| | *GRM7* | 889.54 ± 5.72$^{c}$ | 919.08 ± 6.38$^{b}$ | 967.55 ± 5.27$^{a}$ | 1.70E-12 |
| | *GRM8* | 568.37 ± 27.88$^{c}$ | 720.78 ± 12.26$^{b}$ | 789.53 ± 14.25$^{a}$ | 1.92E-10 |
| | *HRH1* | 704.37 ± 10.73$^{c}$ | 862.75 ± 8.3$^{a}$ | 741.52 ± 7.97$^{b}$ | 1.00E-17 |
| | *HTR1B* | 383.98 ± 38.85$^{b}$ | 380.44 ± 25.83$^{b}$ | 741.42 ± 12.65$^{a}$ | 2.98E-14 |
| | *HTR1D* | 434.39 ± 32.13$^{b}$ | 392.05 ± 33.65$^{b}$ | 791.13 ± 22.79$^{a}$ | 7.62E-14 |
| | *HTR1E* | 626.42 ± 16.22$^{a}$ | 579.41 ± 9.84$^{b}$ | 560.02 ± 12.45$^{b}$ | 0.003242 |
| | *HTR2C* | 360.44 ± 38.47$^{b}$ | 268.26 ± 30.6$^{c}$ | 717.08 ± 22.5$^{a}$ | 1.58E-14 |
| | *HTR4* | 335.95 ± 27.06$^{b}$ | 208.39 ± 22.93$^{c}$ | 598.59 ± 15.53$^{a}$ | 1.93E-17 |
| | *NPY1R* | 781.18 ± 13.49$^{b}$ | 744.88 ± 8.92$^{c}$ | 832.25 ± 6.84$^{a}$ | 4.96E-07 |
| | *NPY2R* | 764.08 ± 26.8$^{a}$ | 278.08 ± 30.99$^{c}$ | 445.66 ± 20.37$^{b}$ | 7.72E-18 |
| | *NPY5R* | 803.27 ± 11.28$^{b}$ | 802.71 ± 5.1$^{b}$ | 890.83 ± 7.03$^{a}$ | 2.17E-11 |
| Neurotransmitter transporter | *SLC1A2* | 1145.76 ± 8.48$^{c}$ | 1169.84 ± 5.78$^{b}$ | 1213.53 ± 7.12$^{a}$ | 7.45E-08 |
| | *SLC1A3* | 1126.62 ± 6.19$^{c}$ | 1171.01 ± 5.99$^{b}$ | 1212.58 ± 7.96$^{a}$ | 1.94E-11 |
| | *SLC5A7* | 462.45 ± 22.56$^{b}$ | 372.9 ± 23.81$^{c}$ | 813.26 ± 28.98$^{a}$ | 8.67E-18 |
| | *SLC6A1* | 1060.1 ± 17.42$^{b}$ | 1066.84 ± 14.9$^{b}$ | 1133.21 ± 15.56$^{a}$ | 0.004225 |
| | *SLC6A11* | 873.74 ± 14.71$^{b}$ | 898.82 ± 8.32$^{ab}$ | 921.46 ± 12.36$^{a}$ | 0.035159 |

| | | | | | |
|---|---|---|---|---|---|
| Neurotransmitter enzyme | *ACHE* | 913.28 ± 12.65$^{b}$ | 902.31 ± 8.99$^{b}$ | 1000.04 ± 9.3$^{a}$ | 8.53E-09 |
| | *DBH* | 142.8 ± 24.9$^{b}$ | 231.02 ± 29.31$^{a}$ | 232.38 ± 24.23$^{a}$ | 0.040051 |
| | *DDC* | 546 ± 30.5$^{a}$ | 438.73 ± 25.96$^{b}$ | 615.04 ± 21.11$^{a}$ | 8.14E-05 |
| | *GAD1* | 1120.74 ± 9.56$^{b}$ | 1144.54 ± 6.56$^{b}$ | 1230.48 ± 9.09$^{a}$ | 1.33E-12 |
| | *GAD2* | 1072.19 ± 11.43$^{b}$ | 1098.65 ± 8.92$^{b}$ | 1249.28 ± 13.82$^{a}$ | 8.01E-16 |
| Neurotrophin | *BDNF* | 824.76 ± 15.71$^{a}$ | 794.94 ± 9.34$^{a}$ | 401.08 ± 30.83$^{b}$ | 5.28E-22 |
| | *NTF3* | 215 ± 30.78$^{a}$ | 253.59 ± 26.55$^{a}$ | 91.13 ± 22.52$^{b}$ | 0.000253 |
| | *NTRK3* | 776.89 ± 18.67$^{a}$ | 682.95 ± 24.05$^{b}$ | 825.92 ± 27.37$^{a}$ | 0.000422 |
| Synaptic vesicle cycle | *CPLX4* | 292.47 ± 23.42$^{a}$ | 211.17 ± 21.21$^{b}$ | 308.37 ± 24.01$^{a}$ | 0.011492 |
| | *RIMS1* | 1079.98 ± 9.96$^{b}$ | 1138.92 ± 6.79$^{a}$ | 1057.74 ± 6.38$^{c}$ | 2.70E-09 |
| | *SNAP25* | 1362.91 ± 5.13$^{c}$ | 1402.06 ± 6.05$^{a}$ | 1380.86 ± 5.16$^{b}$ | 4.40E-05 |
| | *STXBP1* | 1160.96 ± 7.83$^{b}$ | 1208.42 ± 7.97$^{a}$ | 1188.04 ± 9.67$^{a}$ | 0.001635 |
| | *SYT1* | 1178.5 ± 13.27$^{a}$ | 1191.9 ± 14.02$^{a}$ | 1091.12 ± 23.84$^{b}$ | 0.000416 |
| Synaptogenesis marker | *DLG4* | 1177 ± 7$^{c}$ | 1217.53 ± 7.09$^{b}$ | 1249.69 ± 6.62$^{a}$ | 6.29E-09 |
| | *GAP43* | 1184.14 ± 20.49$^{b}$ | 1258.28 ± 13.62$^{a}$ | 1277.53 ± 17.1$^{a}$ | 0.001178 |
| | *SYN1* | 1218.65 ± 4.69$^{b}$ | 1259.07 ± 6.04$^{a}$ | 1193.45 ± 8.45$^{c}$ | 2.00E-08 |
| | *SYN2* | 1077.62 ± 10.44$^{a}$ | 1069.04 ± 8.46$^{a}$ | 1020.33 ± 10.72$^{b}$ | 0.000353 |
| | *SYN3* | 813.8 ± 15.38$^{b}$ | 814.16 ± 6.88$^{b}$ | 937.92 ± 7.19$^{a}$ | 8.80E-13 |

**Only genes with significantly different expressions between brain tissue types are shown. mRNA counts data were analysed via one-way ANOVA with posthoc Fisher's Least Significant Difference. $^{a\ c}$ Values with different superscript letters in the same row differ (FDR < 0.05). Values are represented as mean ± standard error of the mean. FDR, false discovery rate; n, number of samples*

The mRNA abundance of genes encoding for synaptogenesis (*DLG4*, *GAP43*, and *SYN3*) was higher in the striatum than in the prefrontal cortex and hippocampus. In contrast, synapsin isomer (*SYN1* and *SYN2*) gene expression levels were either higher in the prefrontal cortex or similar to the hippocampus compared to the striatum. In addition, the expression levels of neurotrophin genes (*BDNF* and *NTF3*) and synaptic vesicle cycle genes (*SNAP25*, *STXBP1*, *SYT1* and *RIMS1*) except for *CPLX4* were, in general, higher in the prefrontal cortex than in striatum and hippocampus. Overall, genes in the striatum showed increased expression compared to the hippocampus and prefrontal cortex (Appendix Figure A8).

### 5.3.2.2 Milk treatment effect

Ruminant milk type affected the brain gene expression profile of piglets in a brain region-dependent manner. *CPLX4* expression was affected by milk treatments in the hippocampus and striatum (Figure 5-2Aa, j). In the hippocampus, *GRIA2* expression was significantly different in response to milk treatments (FDR < 0.05) (Figure 5-2Ab). In the striatum, *ADRA1D*, *CPLX1*, *GABRA1*, *GABRG2, HTR2B*, *NTF3*, *SLC22A1*, and *SLC6A1* expression levels were significantly different in response to milk treatments (FDR < 0.1) (Figure 5-2A). No genes in the prefrontal cortex significantly differed in expression in response to milk treatments (FDR > 0.1) (data not shown). *HTR2B, NTF3,* and *SLC22A1* expressions were not detected in the striatum tissue samples of piglets fed with caprine milk (Figure 5-2A).

The pairwise differential gene expression between milk treatment groups was visualised using a volcano plot (Figure 5-2B). Genes showing differential expression (FC > 1.5 and FDR < 0.05) in the hippocampus and striatum were identified only between the ovine and caprine groups. No significantly different genes were identified between ovine vs bovine and caprine vs bovine milk groups (data not shown). In the hippocampus, the expression

levels of two genes (*SLC6A1* and *GRID2*) were decreased by > 1.5-fold in the ovine milk group, while *GRIA2* and *RIMS1* increased by > 1.5-fold in the ovine milk group compared to the caprine milk group. The expression levels of seven genes (*CPLX1, GRID2, GRM2, RAB3A, STX3, SLC6A1* and *SLC6A11*) in the striatum were decreased by > 1.5-fold in the ovine milk group compared to the caprine milk-fed piglets. On the other hand, the expression levels of five genes (*ADRA1D*, *GRIA2*, *HTR2B*, *NTF3*, and *SLC22A1*) in the ovine milk group were increased by > 1.5-fold compared to the caprine milk group.

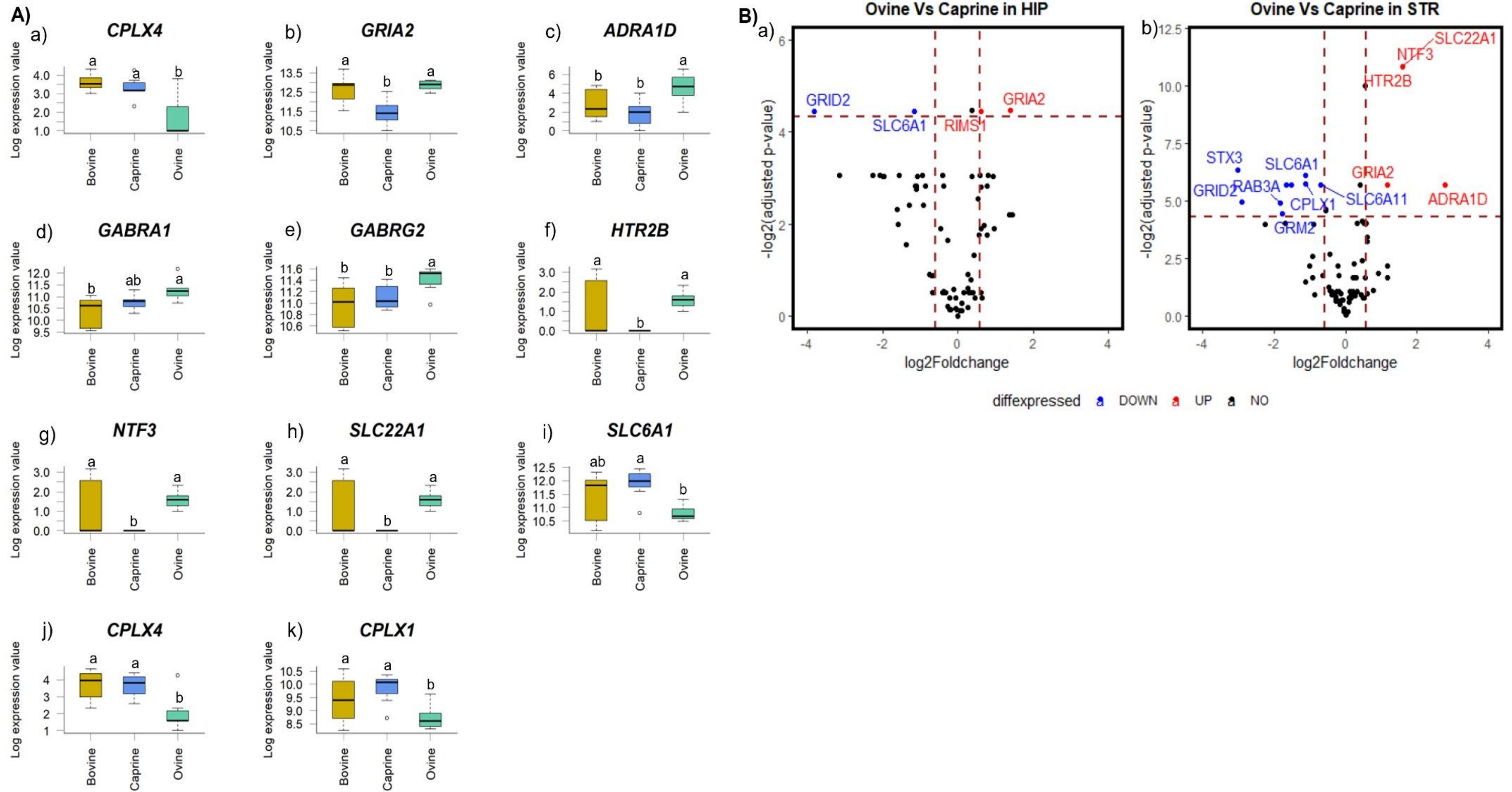


**Figure 5-2 Differential expression of genes in the brain tissues of piglets fed milk from bovine, caprine or ovine species.**

A) Boxplot showing the genes with significant changes in expression levels in a-b) hippocampal and c-k) striatal tissues of piglets fed milk from bovine, caprine or ovine species. The black line in each box indicates the median value, the height of the box represents the interquartile range, and the whiskers

of each box indicate the most extreme values within 1.5 times of the interquartile range. Outliers are shown as individual circles. Milk treatments with different letters differ significantly (FDR < 0.05 for hippocampal genes and FDR < 0.1 for striatal genes). B) Volcano plot showing a pairwise comparison of the differential expression of genes between milk treatment groups a) ovine versus caprine in the hippocampus and b) ovine versus caprine in the striatum. The dotted vertical line indicates fold-change value ± 1.5, and the dotted horizontal lines indicate FDR adjusted P-value < 0.05 threshold. Scattered points represent genes: black points indicate genes that are not differentially expressed, blue points indicate genes that are significantly lower in expression, and red points indicate genes that are significantly higher in expression. Only statistically significant genes (identified using a t-test) are labelled. HIP, hippocampus; STR, striatum; FDR, false discovery rate.

### 5.3.2.3 Milk nutrient intake and brain gene expression correlation

In this study, piglets received different volumes of milk to match the protein intake between milk groups from PND14 or 15, resulting in different amounts of milk nutrients being fed to the piglets (Appendix Table A5). Hence, a correlation analysis was performed, irrespective of the milk treatment, to identify whether the amount of milk nutrient intake influenced brain gene expression. Spearman correlation analysis identified potential relationships between protein, fat, lactose, energy, and dry matter intakes of the milk (from PND 9 or 10 onwards) from different ruminant species and the expression levels of the 77 genes of the individual brain regions (Figure 5-3).

In the hippocampus, 23 positive and one negative correlations existed between milk nutrient intakes and gene expression levels. Significant positive correlations ($P < 0.05$, $rho > 0.5$) were identified between *NPY5R* and fat intake (rho = 0.57), *SLC5A7* and protein intake (rho = 0.58), *SLC5A7* and fat intake (rho = 0.51), *VAMP2* and protein intake (rho = 0.52), *VAMP2* and fat intake (rho = 0.65), *VAMP2* and energy intake (rho= 0.62), and *VAMP2* and dry matter intake (rho = 0.57). No significant negative correlations ($P > 0.05$ or $rho < -0.5$) were found in hippocampal gene expression and milk nutrients (Figure 5-3A).

There were 17 positive and 4 negative correlations between milk nutrient intakes and gene expression levels in the prefrontal cortex. In addition, a significant positive correlation was identified between *GRIA2* and fat intake (rho = 0.51), *GRIA2* and energy intake (rho = 0.54), and *GRIA2* and dry matter intake (rho = 0.54). No significant negative correlations were found between prefrontal cortex gene expression and milk nutrients (Figure 5-3B).

In the striatum, there were 22 positive and 11 negative correlations between milk nutrient intakes and gene expression levels. A significant positive correlation was identified between *CPLX4* and lactose intake (rho = 0.57), GLS and protein intake (rho = 0.51), *RIMS1* and protein intake (rho = 0.62), and *RIMS1* and fat intake (rho = 0.59). Conversely, significant negative correlations were identified in the striatum between *GABRA1* and lactose intake (rho = -0.54), *GABRG2* and lactose intake (rho = -0.57), *GLS* and lactose intake (rho = -0.53), and *SLC22A3* and protein intake (rho = -0.50) (Figure 5-3C).

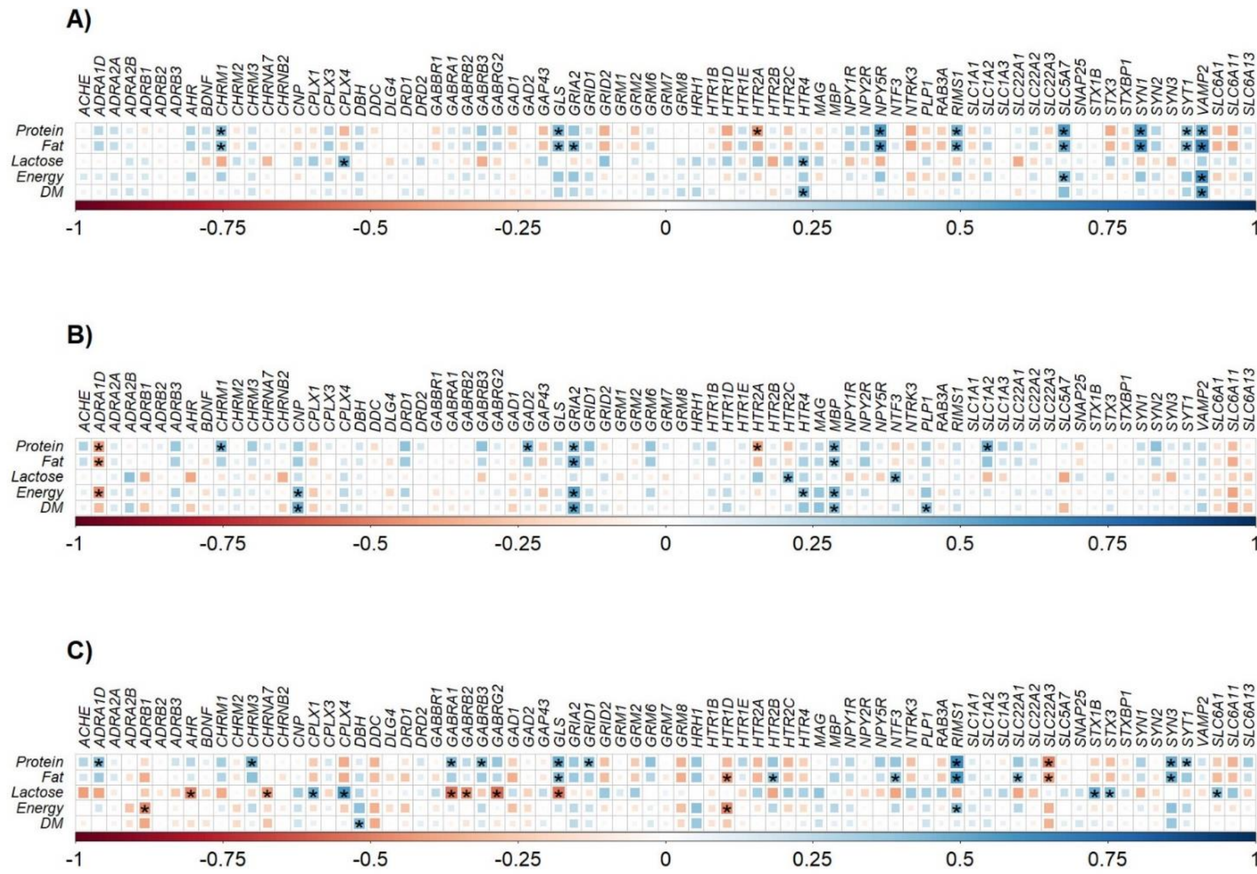


**Figure 5-3 Correlation plot depiction of the Spearman correlations between milk nutrient intakes and gene expression levels in A) hippocampus (n = 22), B) prefrontal cortex (n = 23), and C) striatum (n = 23) tissue samples obtained from piglets fed with milk from bovine, ovine or caprine species.**

The colour and size of the squares indicate the magnitude of the correlation, i.e., blue indicates a positive correlation, and red indicates a negative correlation. Asterisks indicate the significance of correlation ($P < 0.05$). The legend at the bottom of each correlation plot shows the correlation coefficients with their corresponding colours. DM, dry matter; n, number of samples used for correlation.

## 5.4 Discussion

This study is the first to show the effects of feeding whole milk from different ruminant species on the expression of genes in brain regions associated with cognitive function in piglets at approximately PND 21, a well-recognised model of human infant brain development (Guilloteau et al., 2010; Mudd and Dilger, 2017). The results outlined in this study demonstrated that different milk treatments influenced the expression of specific genes encoding for cognitive functions within the brain, and some of these gene expression changes were associated with nutrient intake.

### 5.4.1 Brain-region gene expression differences

Differences in gene expression were observed between brain regions, regardless of the milk treatments. These differences most likely reflect the developmental heterogeneity and functional differences between brain regions (Knickmeyer et al., 2008). In this study, genes encoding neurotransmitter receptors, enzymes, and transporters in the striatum had increased expression levels compared to the hippocampus and prefrontal cortex, suggesting more neurotransmission in the striatum to serve its functional needs. Similarly, in the prefrontal cortex, the genes associated with myelination showed decreased expression levels, which could be explained by the late development of the prefrontal cortex compared to the other regions (Huttenlocher and Dabholkar, 1997; Barkovich, 2005).

### 5.4.2 Brain gene expression differences between different milk

Milk effects on gene expression were brain region-specific. Hippocampal and striatal gene expression profiles were affected by milk treatments, whereas no effect was observed in the prefrontal cortex. One reason for such an effect may be differences in the rate of myelination for different brain areas. Myelination progresses from caudal to

cephalad, i.e., subcortical brain areas (hippocampus, striatum) would be myelinated before cortical area (e.g., prefrontal cortex) (Barkovich, 2005). The myelin sheath is mainly composed of lipids (Barkovich, 2005), and studies with human infants and rodent models have shown that dietary lipids (e.g., SM and fatty acids) are positively associated with myelination (Salvati et al., 1996; Deoni et al., 2018). As lipids are an important source of nutrients in milk (Koletzko, 2017), regardless of the species, it is plausible that consumed lipids can influence myelination, but how remains unknown. However, as the prefrontal cortex is the last to get myelinated compared to the hippocampus and striatum (Volpe, 2000; Barkovich, 2005), it can be speculated that the requirement of lipids for myelin synthesis in the prefrontal cortex would not be as necessary during the initial three months of life as for other brain regions, hence the effect.

In the hippocampus and striatum, genes related to the synaptic vesicle cycle, glutamatergic, GABAergic, serotonergic, and adrenergic transmission, and neurotrophin signalling were most affected by the ruminant milk treatments. Other studies have shown that lower gene expression levels in the hippocampus and striatum associated with these processes were implicated in behaviour changes involving memory impairment in rodent models (Mathew et al., 2010; Tellez et al., 2012; Xie et al., 2015; Ramos-Miguel et al., 2017; Korz et al., 2021). However, the present study did not ascertain any functional relevance of these changes, i.e., whether the level of changes in gene expression between milk treatments was sufficient to change brain function.

Notable differences in gene expression levels were only observed between ovine and caprine milk treatments. In contrast, gene expression levels between bovine and caprine or bovine and ovine milk treatments were similar. These differences in gene expression patterns might be explained by differences in nutrient concentration between ruminant milk treatments (Appendix Table A5). Ovine milk has a distinct nutrient profile with a

higher protein, fat, energy and dry matter content than caprine and bovine milk, as shown in Appendix Table A5 and other studies (Barlowska et al., 2011; Claeys et al., 2014).

However, in this study, piglets received a milk diet balanced for protein content from PND 14 or 15, changing the volume of each milk fed to piglets, resulting in varying amounts of other milk nutrients consumed (Appendix Table A5). For instance, piglets in the ovine milk group received more fat than other milk groups, considering the per day average nutrient intake of the entire study period (Appendix Table A5). It is noteworthy that even if the protein contents were matched between the milk groups, piglets received a different amount of proteins in their milk diets per day on average (Appendix Table A5). This result is because milk nutrient intake was calculated considering the whole study period (except the first three days), which included the days when milk was not balanced for protein content (before PND 14 or 15). Hence, based on the study design, the observed differences in the gene expression levels between ovine and caprine milk groups could not be ascribed to the nutrient concentration difference in the milk. Instead, the effects could be attributed to the amount of each nutrient in the milk intake of piglets.

Differences in composition of fatty acids (Park et al., 2007; Felice et al., 2021), amino acids (Claeys et al., 2014; Rafiq et al., 2016), and oligosaccharides (Van Leeuwen et al., 2020; Shi et al., 2021) also exist between ruminant milk types. For instance, ovine milk has higher Trp and glutamate amino acids than caprine milk, as reported in other studies (Rafiq et al. 2016; Claeys et al. 2014) and Appendix Table A4. Based on the study design, specific amino acids (e.g., Trp, glutamate acids) intakes were also higher in the piglets fed ovine milk than in piglets fed caprine or bovine milk (Appendix Table A4). Interestingly, dietary amino acids are absorbed and metabolised in the gut mucosa and liver and subsequently released in the systemic circulation, where selected amino acids (e.g., Trp, phenylalanine) can cross the BBB and act as precursors for the synthesis of

neurotransmitters in the brain (Zaragozá, 2020). So, it is plausible that the piglets in the ovine milk group would have increased mRNA abundance of the receptor for specific neurotransmitters in the brain to facilitate binding of the neurotransmitter produced through amino acid metabolism. For instance, for 5-HT (*HTR2B*) and glutamate (*GRIA2*) receptor genes, ovine milk-fed piglets showed increased expression levels in the hippocampus and striatum compared to the caprine milk group, suggesting these effects could be a result of differences in amino acid intake between milk treatments. However, the concentration of many amino acids was positively and negatively correlated with the expression of individual genes in the brain areas (Appendix Figure A9). These observations suggest that an effect on the neurotransmitter receptors' gene expression might be due to the protein intake rather than specific amino acid intake.

A previous study had shown that feeding diets with high saturated fatty acids before and during early postnatal periods was associated with decreased expression of hippocampal neurotrophin (*BDNF*), genes encoding for synaptic vesicle proteins (*SYN*), and glutamatergic receptor (*GRIN2B*), as well as learning and memory deficits in the rats (Page and Anday, 2020). Therefore, piglets fed the ovine milk would have consumed higher amounts of total fat and lower amounts of saturated fatty acids, agreeing with results reported in other studies (Park et al., 2007; Felice et al., 2021). Therefore, it would be expected that the genes associated with these processes would have higher expression in the ovine milk group. However, in the current study, genes associated with similar processes were either higher (*NTF3*, *GRIA2*) or lower (*CPLX4*, *CPLX1*) in the ovine milk group than in the caprine milk group. This finding suggests that the milk fat constituents may regulate different genes differently.

### 5.4.3 Milk nutrient intake association with brain gene expression

Milk lactose was correlated with the striatal *CPLX4*, *GABRA1*, and *GABRG2* mRNA counts, and expression of these genes differed between milk treatments. This finding suggests a possible role of lactose intake on specific brain gene expression, but no studies have explored this association. This correlation could be explained by the absorption of lactose and indirectly through microbial lactose fermentation. Lactose in the small intestine is broken down into glucose and galactose and subsequently absorbed into the bloodstream for energy. A study has shown that rats can metabolise glucose and galactose to amino acids (glutamate, glutamine, and GABA) in the brain (Roser et al., 2009). Thus, the change in amino acid (or neurotransmitter) levels in the brain (not measured here) might have influenced its receptor expression. Another study involving *in vitro* fermentation of skimmed milk showed that *Levilactobacillus brevis* co-cultured with *Streptococcus thermophilus* produces GABA neurotransmitters by utilising lactose as the primary source of carbon (Xiao et al., 2020), albeit a limited amount of lactose would be available for large intestinal fermentation *in vivo*. Other studies suggested that circulating GABA can cross the BBB to influence brain function (Al-Sarraf, 2002; Shyamaladevi et al., 2002).

### 5.4.4 Strengths and limitations

The application of NanoString technology for analysing brain gene expression is one of the main strengths of this study. This method detected the true abundance of mRNA in the samples via hybridisation of colour-coded probes and offers an amplification-free detection of mRNA (Wang et al., 2016). Other methods, like RNA sequencing, require enzymatic reactions like reverse transcription and polymerisation, making the measurement indirect; it also does not provide accurate mRNA quantification (Conesa et

al., 2016). However, NanoString cannot provide any information on genes not included in the probe set used.

The piglets in this study received the diet on a per kg BW basis, and these differences in milk intake may have resulted in some differences in the brain gene expression as heavier animals received more milk than lighter animals. It is also important to note that other nutrients in milk (e.g., oligosaccharides, fatty acids) than those analysed could have explained associations between the milk treatments and brain gene expression changes. Unlike some animal studies, this study removed the confounding effect of uncontrolled food intake by fasting the piglets and euthanising them at the same post-feeding time, making this a more controlled study.

Another limitation is that the gene expression responses were only studied in male piglets. However, a study has shown that the expression of *BDNF* in the hippocampus in GF mice was sex-dependent (Clarke et al., 2013). Hence, including female piglets in the study would have been informative, but it will have required more piglets per treatment beyond the scope of this study.

It is acknowledged that using a reference group, i.e., piglets fed with sow’s milk showing basal expected brain gene expression would have been relevant to compare with those fed with other species' milk. However, the brain samples were collected from a study that aimed to compare structural changes in bovine with non-bovine (ovine and caprine) raw whole milk on digestion of piglets in early postnatal life, and using piglets fed with sow’s milk was not required for the experiment. Therefore, future studies should compare, where possible, mRNA gene expression levels of piglets fed with sow’s milk and those fed with milk from other species.

Furthermore, changes in gene expression do not necessarily translate to changes in protein expression or physiological function. However, they are the essential first step of most biological processes. Therefore, future studies should measure changes in the abundance of proteins involved in cognitive function and associated behaviours. These data would help understand whether the observed gene expression changes in response to the consumption of bovine, caprine and ovine milk would contribute to changes in brain function and behaviour in early postnatal life.

## 5.5 Conclusion

This study is the first to investigate the effects of consuming whole milk from different ruminant species on the expression of genes related to cognitive function in the brain in the early postnatal life of piglets as a model for human infants. Different ruminant milk treatments, consumed at different volumes to balance protein intake, altered the expression of neurotransmission genes important for cognitive development in early postnatal life. This study also highlighted a brain region-specific milk effect, suggesting the importance of studying individual brain regions rather than the whole brain as one homogenous organ. While ruminant milk is not an alternative to human milk, this study provides novel insights into the biological impact of whole milk from different ruminant species, which upon further research, can be applied to design nutritionally-advanced infant formula.

# Chapter 6

# Integration of changes in brain and blood variables in response to milk treatments in early postnatal life

# Abstract

Evidence from previous studies has suggested that peripheral blood plasma metabolites influence brain functions in the early postnatal period. However, whether ruminants' milk treatment-associated plasma metabolites profile influences brain development in the early postnatal period remains unknown. This chapter aims to investigate whether changes in plasma lipid intensity profile observed in response to ruminants' milk treatment correlates with relative intensity profile of lipids in the brain areas associated with cognitive development (hippocampus, prefrontal cortex, and striatum) in the early postnatal period. Multi-omics data integration analysis was used to identify correlations between these data sets. Positive and negative correlations ($r > 0.7$) were primarily identified between plasma PC and TG relative intensities and brain phospholipids (e.g., PC, PS, and PE). Hippocampal and striatal lipids were both positively and negatively associated with plasma lipids. In contrast, prefrontal cortex lipids were only negatively associated with plasma lipids. Furthermore, the integration analysis of plasma and brain lipids showed that the bovine milk treatment group was well discriminated from the ovine and caprine milk groups. The associations between changes in brain lipids and circulating blood plasma lipids in piglets consuming bovine, caprine, or ovine milk suggest differences in lipid metabolism in the gut, warranting further research.

## 6.1 Introduction

Human breast milk is the best source of infant nutrition and promotes optimal growth and development of infants. However, when breast milk is limited or unavailable, infant formula based on milk from other species is used as an alternative source of infant nutrition. In this context, milk from ruminant species like bovine, caprine and ovine are increasingly used. However, they vary in their composition. For instance, the protein, fat,

and energy content of ovine milk are higher than that of bovine and caprine milk, which are more similar (Park et al., 2007; Barlowska et al., 2011; Claeys et al., 2014). Additionally, the oligosaccharide content of caprine milk is higher than that of ovine and bovine milk, and its profile is more similar to that of human breast milk (Van Leeuwen et al., 2020; Shi et al., 2021).

Consumption of other ruminant milk has been suggested to impart different physiological responses in the host. For instance, *in vitro* studies have shown that fermentation of pre-digested ovine, caprine, and bovine milk using an infant faecal inoculum showed that the microbial composition of fermentation samples differs between the milk treatments (Ahlborn et al., 2020; Gallier et al., 2020a). Another study in women of age > 20 years showed that amino acid concentration in the peripheral blood plasma varied after ovine or bovine milk consumption (Milan et al., 2020). Although studies have not explored the effects of ruminant milk consumption on brain function, studies have shown that differences in sphingolipid and oligosaccharide levels in human milk and formula were associated with cognitive development in infants (Deoni et al., 2018; Berger et al., 2020; Fleming et al., 2020). These observations suggest that differences in nutrient composition between ruminant milk may also influence brain development.

The potential role of circulatory plasma metabolites in the brain functions in physiological and pathological conditions in the neonatal period has been extensively explored (Wiedeman et al., 2018; Sotelo-Orozco et al., 2020; Friedes et al., 2022). For instance, a study in healthy infants of 12 to 14 months of age showed that higher plasma betaine, a choline metabolite, was positively associated with increased visual and motor skills development at two years of age (Wiedeman et al., 2018). However, whether the diet- and/or gut-derived metabolites found in circulatory blood plasma are associated with the

metabolites and expression of genes in the brain areas crucial for cognitive development remains to be explored.

Chapters 3, 4 and 5 report new findings on the effects of bovine, caprine and ovine milk treatments on the peripheral blood plasma metabolome, brain metabolome and brain gene expression in piglets as a model for human infants. Chapters 3 and 4 showed that milk treatments primarily influenced lipid (non-polar) metabolites. Milk treatments affected a few (< 10) known polar metabolites, with most metabolites remaining unidentified. Chapter 5 showed that milk treatments influenced a few (< 10) gene expressions in the brain.

Hence, based on these findings, changes in the peripheral blood plasma lipid profiles in response to different ruminants' milk treatment were hypothesised to be associated with the changes in the lipid profiles in the brain. Therefore, this chapter aimed to identify the correlations between plasma (Chapter 3) and brain (Chapter 4) lipid relative intensities using the multi-omics data integration method. Plasma and brain polar metabolomic and brain gene expression data sets were not used for data integration due to a smaller number of significant features identified in response to milk treatments.

## 6.2 Materials and methods

Integration of plasma lipidome, brain lipidome, and brain mRNA counts data was conducted using the Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) function (Singh et al., 2019) from the `mixOmics` package of R (version 4.1.1). DIABLO is a supervised method that guides the data integration process by identifying the correlated variables from data sets to predict outcomes.

Initially, the correlations between data sets were calculated using the PLS function of the mixOmics package, and these values were inputted into a data-driven weighted design matrix (Appendix Table A6). After setting the design matrix, the DIABLO model was first fit using five components selected manually, and the model's performance was checked using leave-one-out-cross validation (LOOCV). Next, the number of components was selected based on the lowest balanced error rate and centroid distance across the components, followed by manual feature tuning and selection (Appendix Table A7).

A final multiblock sPLS-DA model was achieved using selected components and features and validated using LOOCV. The overall diagnostic accuracy of the model was checked using the area under the receiver operating characteristic (ROC) curve. The area under the ROC curve was calculated for each data set individually, and P-values were calculated using the Wilcoxon test for pairwise comparisons of milk treatment groups with P-values $< 0.05$ considered significant. The area under the ROC curve values ranges from 0-1, with 1 indicating a perfectly accurate model; 0.8-0.9, excellent model; 0.7-0.8, good models, and value 0.5 represents no discriminatory ability (Singh et al., 2019).

Clustered image heatmaps were used to visualise the signature pattern of each sample. Circos plots were used to visualise the correlation between variables from different data sets, and a cut-off was chosen as $r = 0.7$, representing a strong positive or negative correlation.

## 6.3 Results

### 6.3.1 Correlations between plasma and brain lipid metabolites

DIABLO analysis identified strong correlations between plasma lipidome and hippocampal lipidome (r=0.78), plasma lipidome and prefrontal cortex lipidome (r=

0.72), and plasma lipidome and striatal lipidome (r=0.83) data sets. DIABLO analysis showed that the bovine milk treatment group was well discriminated from the ovine and caprine milk groups in all three data sets (Figure 6-1). Subsequent hierarchical clustering analysis of the integrated plasma and brain lipid metabolite data sets confirmed the clustering pattern observed by the DIABLO sample plots, i.e., three distinct clusters of samples based on milk treatments were observed (Figure 6-2). All the models achieved an area under the ROC curve over 0.78 ($p < 0.05$), indicating good specificity and sensitivity (Appendix Figure A10-A12)

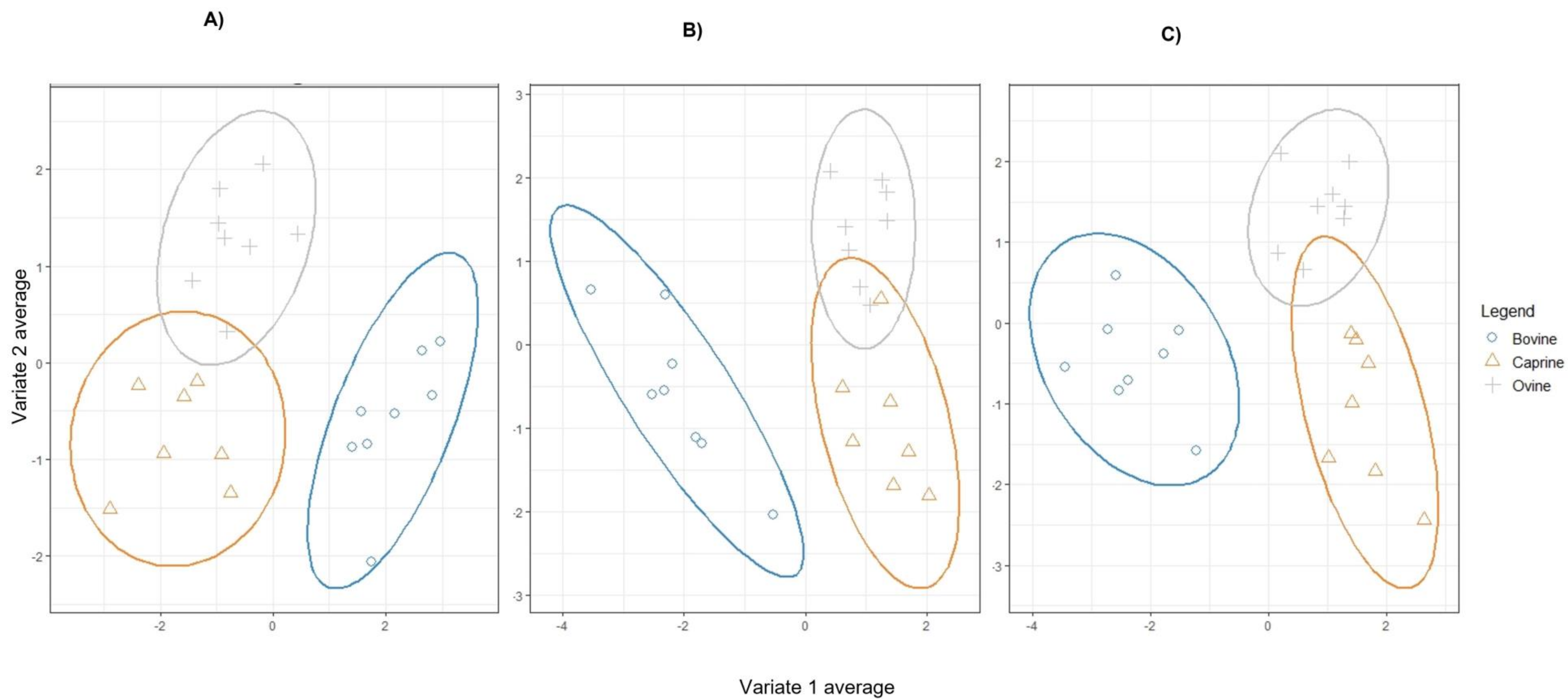


**Figure 6-1 Sample plots from DIABLO analysis applied on plasma lipid relative intensities and A) hippocampal B) prefrontal cortex C) striatal lipid relative intensities.**

The plot is represented as the average of all components from the blocks (plasma and brain data sets).

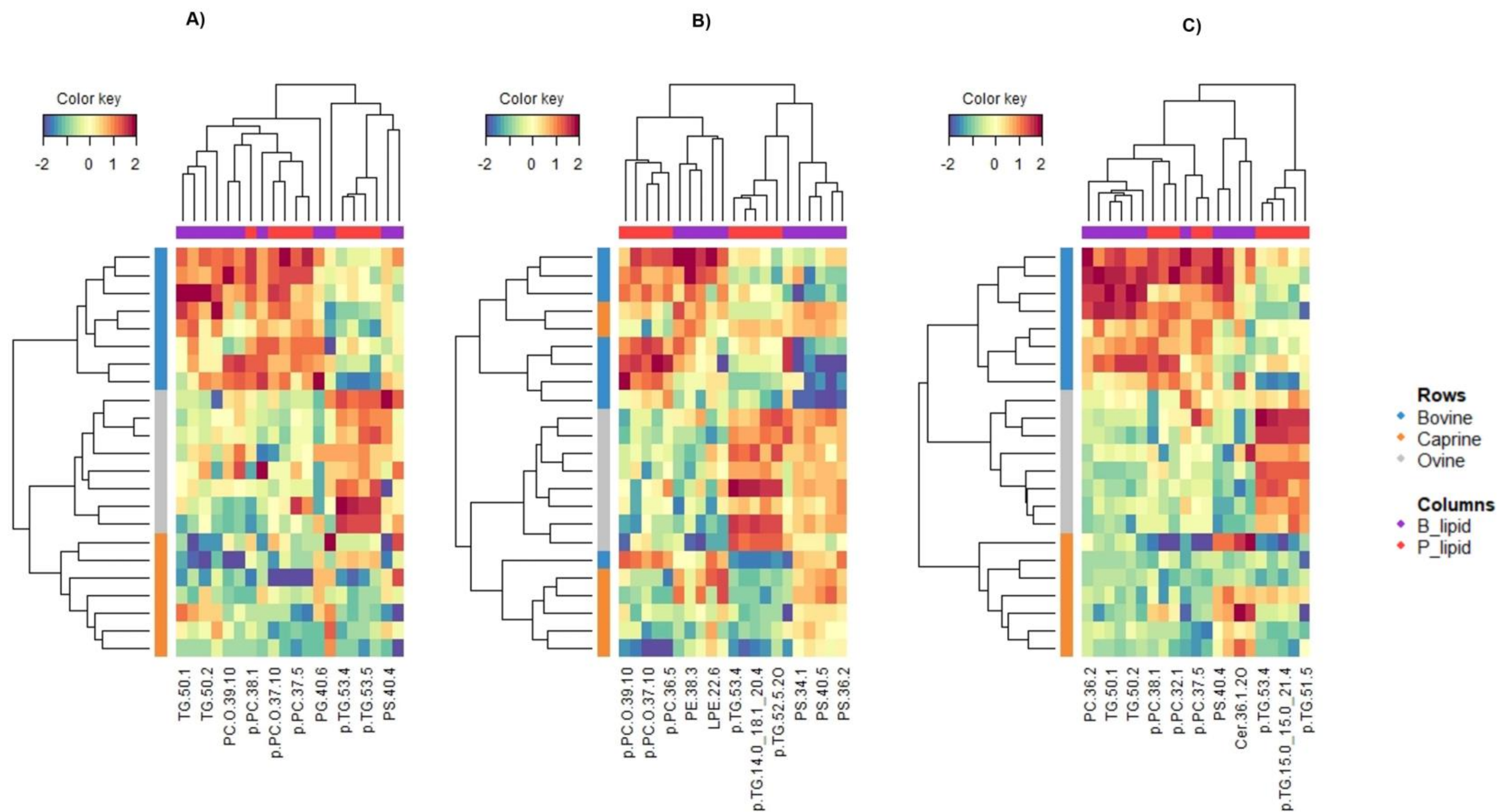


**Figure 6-2 Clustered Image Map for the variables selected by multiblock sPLS-DA performed on the plasma lipid relative intensities and A) hippocampal, B) prefrontal cortex, and C) striatal lipid metabolite relative intensities.**

The samples are represented in rows (indicated by their milk treatment on the left-hand side of the plot) and selected features in columns (indicated by their data type at the top of the plot).

Strong positive correlations were identified between the relative intensities of hippocampal TG and PS species and plasma PC species (Figure 6-3A). For example, Hippocampal TG 48:1 was positively correlated with plasma PC-O-37:10, PC 36:5, and PC 37:5. Hippocampal TG 50:2 was positively correlated with plasma PC-O-37:10, PC 36:5, PC 37:5, PC 32:1, and PC 38:1. Hippocampal PS 36:1 was positively correlated with plasma PC 32:1 and PC 36:5. Hippocampal TG 48:0 was positively correlated with plasma PC 32:1. Hippocampal TG 50:1 was positively correlated with plasma PC 37:5, PC-O-37:10, and PC 32:1. Strong negative correlations were identified between hippocampal phosphatidylglycerol (PG) PG 40:6 and plasma TG (53:4, 53:5, 52:5;2O, and 52:6) (Figure 6-3A).

Strong negative correlations were identified between the relative intensities of prefrontal cortex PS, PC, LPC and LPE species and plasma PC and TG species (Figure 6-3B). Prefrontal cortex PS 38:4 was negatively correlated with plasma PC 32:1, PC-O-39:10, PC-O-37:10, and PC 36:5. Prefrontal cortex PS 40:5 was negatively correlated with plasma PC 38:1, PC 32:1, PC-O-39:10, PC-O-37:10, and PC 36:5. Prefrontal cortex PC-O-37:9 was negatively correlated with plasma PC 38:1, PC 32:1, PC-O-37:10, and PC 36:5. Prefrontal cortex PS 36:2 was negatively correlated with plasma PC 38:1, PC 32:1, PC-O-39:10, PC-O-37:10, and PC 36:5. Prefrontal cortex PS 34:2 was negatively correlated with plasma PC 32:1, PC-O-37:10, and PC 36:5. Prefrontal cortex LPE 22:6 was negatively correlated with plasma TG 53:4, TG 53:5, TG 52:5;2O and TG 52:6. Prefrontal cortex LPC 16:0 was negatively correlated with plasma TG 52:6. TG 53:5, and TG 14.0_18.1_20.4.

Strong positive correlations were identified between striatal PS, PC, and TG species and plasma PC species (Figure 6-3C). Striatal PS 38:4 was positively correlated with plasma PC 36:5. Striatal TG 48:1, TG 52:2, TG 48:0, TG 50:1, and 50:2 was positively correlated

with plasma PC 38:1, PC 32:1, PC-O-37:10, PC 36:5 and PC 37:5. Striatal PC 36:2 was positively correlated with plasma PC 38:1, PC-O-37:10, PC 36:5 and PC 37:5. Strong negative correlations were identified between plasma TG species and striatal PE, and CER species (Figure 6-3C). Striatal Cer 36:1; 2O was negatively correlated with plasma TG 53:4, TG 53:5, TG 14.0_18.1_20:4, TG 51:5, and TG 15.0_15.0_21.4. Striatal PE 38:4 was negatively correlated with plasma TG 53:4, TG 53:5, TG 14.0_18.1_20:4, and TG 15.0_15.0_21.4.

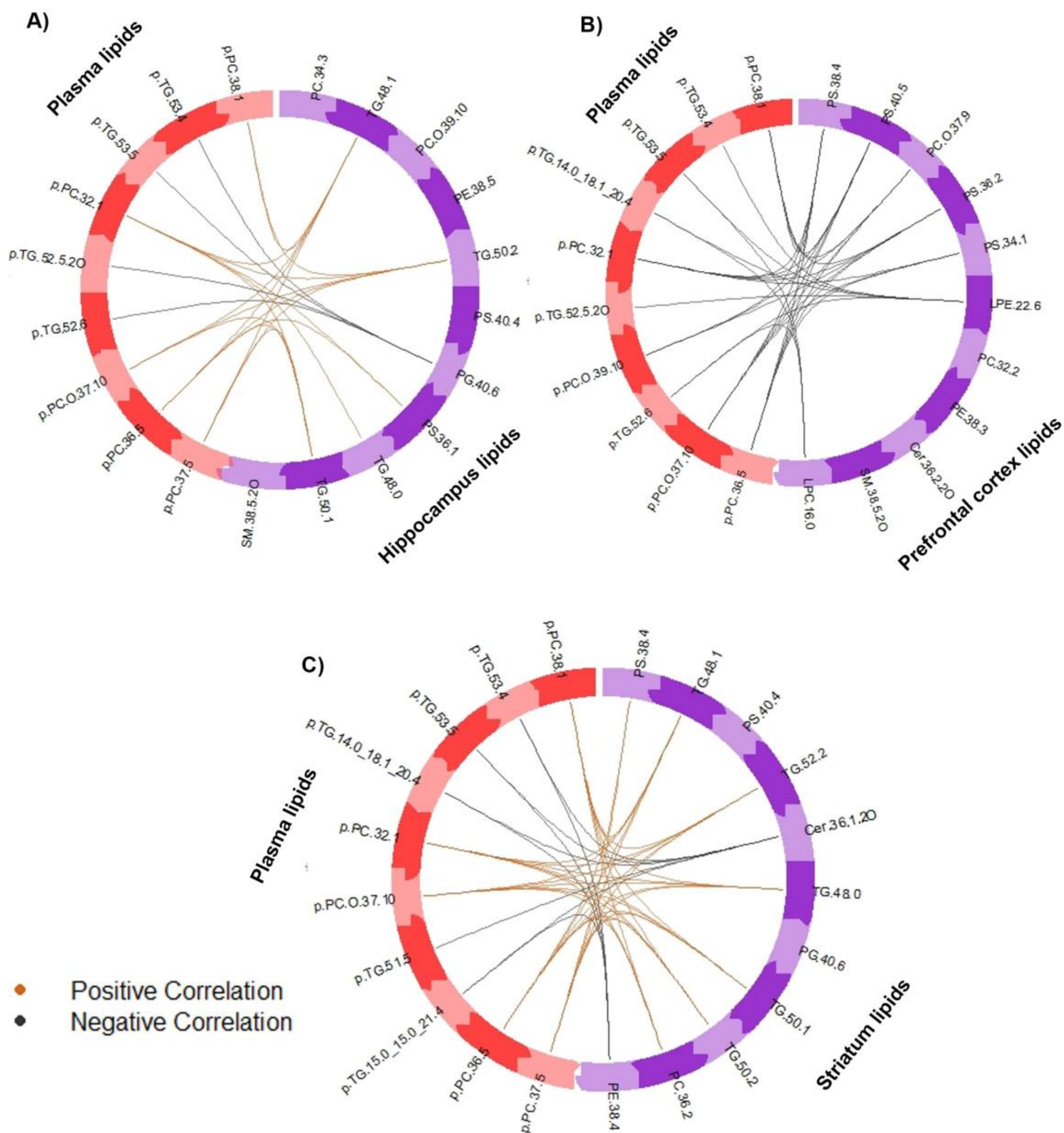


**Figure 6-3 Circos plot output for DIABLO representing the associations between plasma lipid and A) hippocampal, B) prefrontal cortex C) striatal lipid relative intensity data sets, irrespective of milk treatments.**

The inner lines indicate positive (red) and negative (black) correlations between variables. Correlation cut-off was set to r = 0.7, and the plot was generated using the first two components of the model. The middle ring indicates the variables within the data types: plasma lipids (red) and brain lipids (purple). Plasma lipid species are preceded with the letter p.

## 6.4 Discussion

The integration analysis conducted in this chapter showed associations between lipid features in brain areas crucial for cognitive development (Chapters 4) with changes in circulatory blood plasma lipid metabolites (Chapter 3) elicited by consumption of bovine, caprine, or ovine milk treatment in piglets as a model for human infants.

The results showed that the changes in the plasma lipid relative intensities were highly correlated with brain lipid relative intensities in a brain-region-dependent manner. The results also highlighted that the plasma and brain lipid variables could be used to discriminate the milk treatments.

### 6.4.1 Associations between plasma and brain lipid metabolites

PC and TG were the two main plasma lipid classes positively and negatively correlated with brain phospholipids (PS, PC, PE, PG, LPE and LPC) and TG, irrespective of the brain regions. Interestingly, these lipid species in plasma and brain tissues primarily consist of PUFA (e.g., PC 36:5, PC 37:5, TG 53:5, TG 53:4). PUFA are crucial for brain development and function, but they cannot be synthesised in the brain and must be transported to the brain through peripheral blood circulation (Spector, 2001).

The exact mechanism by which plasma lipids influence brain lipids remains unclear. Studies have suggested that PC-linked PUFA could be transported to the brain in the form of high-density lipoprotein that can cross the BBB (Balazs et al., 2004; Koulman et al., 2014). Lipoprotein lipases present in the endothelial cells have been hypothesised to hydrolyse the phospholipids of the lipoprotein-releasing PUFA that are then further transported across into the brain for subsequent metabolism and reassembly into phospholipids (Edmond, 2001; Chen et al., 2008; Mitchell and Hatch, 2011; Pifferi et al., 2021). TG can also cross the BBB (Banks et al., 2018), which like phospholipids, might

be broken down and reassembled into TG or other lipid species in the brain (Chen et al., 2008).

Although the correlation analysis in this study showed an association between plasma lipids and brain lipids, associations between the same lipid species in plasma and the brain were not observed. For instance, while plasma PC 32:1 was not correlated with PC32:1 in any brain region, it was positively correlated with PS 36:1 in the hippocampus. This observation could be due to the endogenous synthesis of different lipid species in the brain using peripheral lipids as precursors. For instance, PS can be converted into PC by the activity of PS-synthase-1 (Kim et al., 2014).

It is important to note that brain and blood samples were collected at 210 min post-feeding. Therefore, the observed changes in the brain metabolites profile were most likely due to the turnover of lipids throughout the study period and not due to the last meal effect, unlike the plasma metabolite profile. Hence, this mismatch could contribute to the lack of identification of similar lipid species in the plasma and brain and their correlation.

### 6.4.2 Milk treatment effect on plasma and brain lipid metabolites

Integration of plasma and brain lipid relative intensity data sets showed that the bovine milk group was discriminated from the caprine and ovine milk groups. Associations between lipid intakes and plasma and brain lipid relative intensities of piglets fed bovine, caprine or ovine milk could not be established in Chapters 3 and 4, most likely due to the complex digestion and absorption of lipids.

However, a study reported differences in the fatty acid inter-positional composition of TG fraction between bovine, ovine and caprine milk (Blasi et al., 2008). For instance, they showed that bovine milk contains higher levels of medium chain fatty acids (e.g., C8:0, C10:0, C14:0) at the sn-2 position of TG compared to the caprine or ovine milk

(Blasi et al., 2008) . Given the sn-2 position of TG suitability for digestion and absorption (German and Dillard, 2006; Innis, 2011), it could be speculated that bovine milk lipids in the current study, might have a higher levels of medium chain fatty acids at the sn-2 position, resulting in efficient digestion and absorption of bovine milk TG and subsequent metabolism into other lipid species like PC and SM, thereby influencing the brain lipid metabolite profile.

### 6.4.3 Strengths and limitations

The strength of this chapter was the use of the mixomics framework for the integration of data sets, which has enabled the identification of selected omics features and a better understanding of the correlation pattern. Also, the data integration method used provided a first filtration step for future knowledge-driven pathway analysis.

A major limitation was that no prior studies were available to validate whether the correlations identified represent a mechanistic relationship between plasma and brain variables or were chance events. Another limitation is the inability to provide holistic insight into diet-mediated early postnatal brain development due to using only lipidomics data sets for integration purposes.

## 6.5 Conclusion

In conclusion, the integrated analysis conducted on the plasma and brain lipidome showed that relative intensities of a subset of lipids were correlated between plasma and brain areas (hippocampus, prefrontal cortex, and striatum) critical for cognitive development. Plasma TG and PC were positively or negatively associated with brain phospholipids in a brain-region-dependent manner. The results highlighted that the plasma and brain lipidomes were robust data sets separating the milk treatment groups, highlighting the importance of these data sets in the early postnatal period.

The correlative analysis provided insights into potential lipid metabolites in the context of dietary treatments that might influence brain development in the early postnatal period. Future studies involving multiple brain omics data sets (e.g., transcriptomics, proteomics) would help create a holistic understanding of ruminants' milk effect on early postnatal brain development.

# Chapter 7

# General Discussion

## 7.1 Thesis discussion

This PhD thesis explored the effects of milk from bovine, caprine and ovine species on early postnatal brain development via gut-derived metabolites using piglets as a model of human infants. The results outlined in the thesis showed that the circulatory blood plasma metabolites, especially non-polar (lipid) metabolites, differed between milk treatments, with higher relative intensities of phospholipids (e.g., PC, saturated TG) in the bovine milk group compared to caprine and ovine milk groups. In addition, the metabolite profile, especially the lipid profile in the brain areas crucial for cognitive development, also differed between milk treatments, with higher relative intensities of lipids (e.g., PC, PS, PE) in the bovine milk group than in other milk groups in the striatum and hippocampus. In contrast, lipids' relative intensities in the prefrontal cortex were higher in the ovine milk group than in other milk groups.

The expression profile of some genes associated with neurotransmission in the hippocampus and striatum also differed between milk treatments, with both increased and decreased gene expression profiles observed in the ovine milk group compared to the bovine and caprine milk groups, whereas the caprine and bovine milk groups showed a similar gene expression profile. Milk treatments did not influence the prefrontal cortex gene expression profile.

Furthermore, the results highlighted that plasma TG and PC were positively associated with the striatum and hippocampus phospholipids and negatively associated with prefrontal cortex phospholipids. A diagram illustrating the main new findings of this PhD research is shown in Figure 7-1.

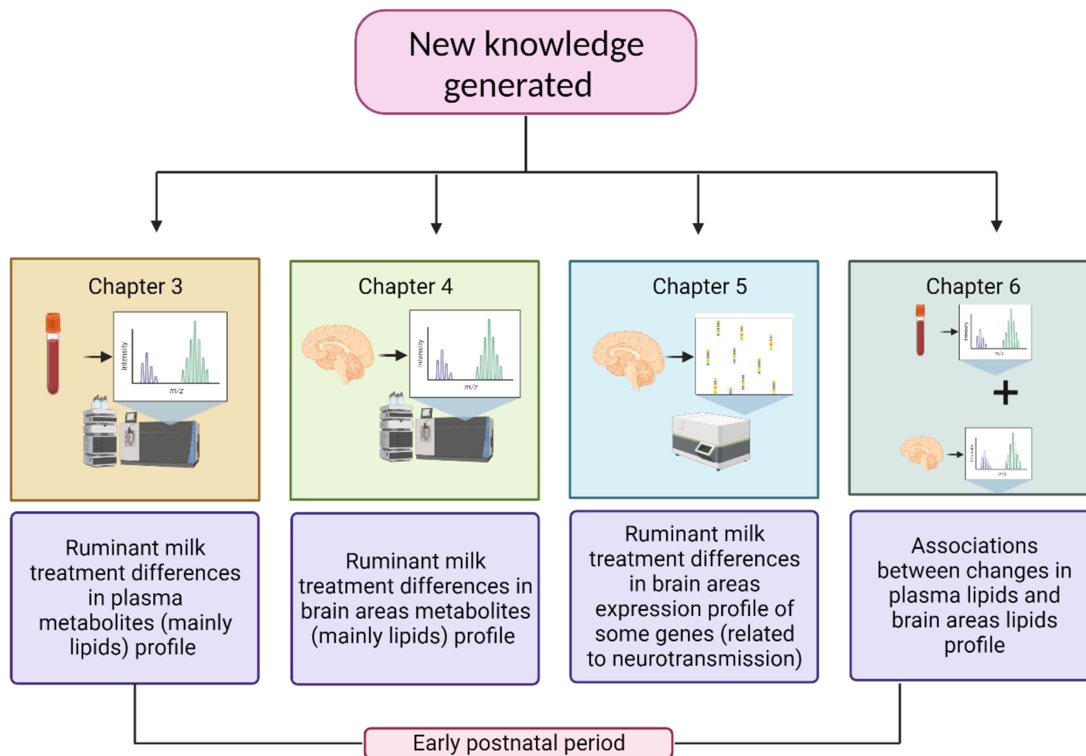


**Figure 7-1 The new knowledge contributed by this PhD research findings**

Figure created using Biorender.com

Until now, no studies have compared the effects of bovine, caprine and ovine milk on these parameters. Instead, studies have focussed on the effects of these milk on digestion and fermentation *in vitro* or a piglet model of the human infant and appearance in peripheral blood circulation in humans. Hence, this PhD research is the first to provide evidence on the impact of bovine, caprine and ovine milk consumption on relative intensities of brain lipid metabolites and expression of genes involved in brain development. Furthermore, the research also established associations between the peripheral lipidome profile and the lipidome profile of specific brain areas, which could help better understand potential metabolites that may influence brain development in the early postnatal period.

In this thesis, piglets were used as a model of human infants because of their greater similarities in gut physiology, gross neuroanatomy, and developmental pattern with humans compared to rodent models. Therefore, findings from piglet models are likely to be better translated to human infants than that from rodent models.

Samples used in this thesis were collected from a study where piglets received different volumes of each milk type to compensate for the similar protein intake between piglets. However, this adjustment resulted in differences in the intake of other nutrients between piglets fed with each milk type. Hence, the nutrient concentration differences between the raw whole milk, as reported by others and in this thesis, becomes less relevant in the context of the responses observed in the peripheral blood circulation and brain tissues, and the focus was given to nutrient intake differences in this thesis.

In the peripheral blood circulation, the relative intensity of many lipids differed between milk treatments, with a higher relative intensity in piglets fed bovine milk (e.g., TG and PC) compared to those fed ovine and caprine milk treatments (Chapter 3). These differential lipid profiles might have resulted from differential lipid intakes between groups. In addition, dietary lipids are broken down into fatty acids and monoglycerides in the small intestinal lumen, then absorbed into the enterocytes for re-esterification and subsequent packing into chylomicrons, which are transported via the lymph to the bloodstream and then to body tissues. This complex process of continuous breakdown and reassembly of lipid species likely increases the variability of lipid species in the plasma, making tracing the correlation with dietary lipid intake challenging, as observed in Chapter 3.

The use of an untargeted LC-MS approach for the analysis of polar and non-polar (lipid) metabolites provided broad coverage of blood plasma and brain metabolomes. However,

annotating these metabolites was more achievable for lipid molecules than polar ones, which remained primarily unidentifiable with available databases. However, the quantification of plasma amino acids in combination confirmed some amino acids (e.g., proline, tyrosine, lysine), which relative intensities in the polar metabolite extract of peripheral blood plasma differed between milk groups. In addition, higher levels of amino acids were found in the ovine milk group compared to other milk groups. However, these concentration differences between the milk treatments were not due to amino acid intakes, as animals were given similar amino acid intakes between milk groups. Hence, the elevated levels of plasma amino acids in response to consuming ovine milk were speculated to occur due to increased and/or more rapid amino acid absorption, as discussed in Chapter 3.

The variations observed in peripheral blood plasma metabolite profiles between ruminant milk treatments might also be due to the difference in lipids and protein metabolism in the gut tissue and liver and gut permeability between milk treatment groups. For instance, a study showed that ileal expression of Trp metabolism (e.g., *TPH1*, *KYNU*, and *SERT*) and nutrient transporters (e.g., *GLUT1* and *PLA2G4*) genes were either increased or decreased in human milk-fed piglets compared to infant formula-fed piglets (Charton et al., 2022). In addition, the author showed that piglets fed with human milk had higher ileal and colonic paracellular permeability than infant formula-fed piglets (Charton et al., 2022). Another study showed that expression of genes (*ACAA2*, *ACACB*, and *SLC27A5*) associated with hepatic lipid metabolism were elevated in response to high-fat cow milk treatment compared to high-fat buffalo milk treatment in a mouse model (Jiang et al., 2022).

Gut microbial metabolism of some dietary nutrients may also influence peripheral blood plasma metabolite profiles between ruminant milk treatments. Studies have shown that

the gut microbiota can also produce and modulate different lipids species (e.g., sphingolipids, phospholipids) and amino acids (Metges, 2000; Yasuda et al., 2020; Lamichhane et al., 2021; Liebisch et al., 2021). Interestingly, *in vitro* studies have suggested differences in gut microbiota profiles between different pre-digested milk types (Ahlborn et al., 2020; Gallier et al., 2020). However, no effect of milk treatments on the ileal and caecal microbiota composition and predictive function was observed in this study (Halliday, 2022). Hence, future studies involving analyses of gut tissue parameters associated with nutrient absorption and metabolism, in particular lipids, and maybe gut microbial metabolism would help interpret the differences in peripheral blood plasma metabolite profiles in response to ruminant milk treatments.

In the brain, the relative intensity of phospholipids and TG primarily differed between milk treatments, with a higher relative intensity of these lipids found in the bovine milk group compared to the ovine or caprine milk group (Chapter 4). The data integration analysis (Chapter 6) found that all these significant brain lipids were positively or negatively correlated with plasma PC or TG. Studies have suggested that peripheral metabolites can influence brain activity mainly by crossing the BBB or interacting with the VN (Balazs et al., 2004; Bravo et al., 2011; Koulman et al., 2014). For instance, choline-containing phospholipids were suggested to cross BBB and participate in the synthesis of neurotransmitters, supplying and maintaining the membrane structure of neurons in the brain (Roy et al., 2022b). Another study in mice showed that vagal sensory neurons, particularly peroxisome proliferator-activated receptors γ, act as a receptor for dietary-derived lipids (Liu et al., 2014). A study in rats has highlighted that stimulation of the peripheral VN influenced the lipid abundance and protein secondary structures in the brain areas, including the striatum and motor cortex (Surowka et al., 2015).

Milk treatments did not show any major effect on the expression of genes in the areas of the brain crucial for cognitive development. Nevertheless, the NanoString gene panel (77 genes) used in this thesis provided important insight into the expression profiles of genes relevant to neurotransmission in the brain in response to milk treatment (e.g., glutamate, GABA, 5-HT, adrenaline and neurotrophin signalling and the synaptic vesicle cycle processes), particularly in the hippocampus and striatum. The inclusion of more genes in the panel or the use of RNA sequencing might have helped identify other significant genes. However, including more genes in the NanoString brain gene panel required prior evidence on selected genes transcript and protein levels, which remains unavailable for most genes in the piglet model. Therefore, RNA sequencing would be a better approach to generate a comprehensive gene expression signature in response to the milk treatments.

Since brain gene expression was mostly unaffected in response to milk treatments in this thesis, correlations between brain gene expression profile and brain metabolome profile were not conducted. However, previous studies have suggested that brain phospholipids are crucial regulators of neurotransmission in the brain. For example, a study has shown that the polar head of the PS resembles the glutamate neurotransmitter that acts as a ligand for the glutamate receptor, regulating the activity of glutamate receptors in the hippocampus (Foster et al., 1982). Additionally, studies have shown that phospholipids regulate the activity of GABA neurotransmitter binding to its receptor (Lloyd and Beaumont, 1980), and acetylcholine release, thereby regulating the GABA and acetylcholine-associated neurotransmission. Hence, an association between brain lipids and brain gene expression might be possible but was not established in this thesis.

Along with lipids, polar metabolites like amino acids also regulate the neurotransmission function, primarily by serving as precursors for neurotransmitter synthesis (Mittal et al., 2017). However, only a few identified polar metabolites in the brain were influenced by

milk treatments. As a result, no correlation analysis was conducted between polar metabolites' relative intensity and gene expression in the brain. Hence, the association between brain amino acids abundance and neurotransmission gene expression between milk treatment groups remains to be elucidated.

Among all the brain regions evaluated in this thesis, striatal metabolite and gene expression features appeared to be most influenced by milk treatments. This diet-mediated brain region effect is a novel observation. Therefore, it is plausible that the striatum is more malleable to dietary changes than the hippocampus and prefrontal cortex in the early postnatal period. This speculation warrants investigation.

## 7.2 Limitations

Before the commencement of the experimental period, piglets had consumed *ad libitum* sow's milk, which makes it challenging to ascertain whether the effects observed at the end of the experimental period were due to the milk treatments alone. However, the potential effect of sow's milk would have been present across milk treatment groups. Another limitation was that no control groups of piglets fed the sow's milk were compared with those fed other species' milk. Biological samples were collected from a study to compare structural changes in ruminants' raw whole milk on piglets' digestion in early postnatal life, which did not require piglets fed with the sow's milk.

Another limitation of this thesis is the unavailability of information on number of litters the animals obtained. Although the primary study aimed to select the healthiest male piglets, it is unclear whether piglets from the same litter were evenly distributed across different milk treatments. As a result, any confounding effect of the litter on the different responses analysed cannot be definitively ruled out. Additionally, using a single timepoint for analyses made it difficult to distinguish whether the responses observed were an

immediate or long-term diet effect due to the continuous turnover of nutrients in the host over the study period.

Furthermore, a premix of vitamins and minerals was added to the spray-dried whole milk powder to ensure all animals received the required nutrients. However, this premix did not consider the difference in vitamins and mineral content between milk treatments, potentially resulting in different vitamin and mineral profiles than the original whole milk profile.

Most importantly, based on the study design, different volumes of each milk were fed to the pigs to balance milk protein intake. However, this limitation allowed for exploring the effects of differences in the nutrient intake of pigs on brain and blood plasma variables.

Milk amino acid and lipid contents were shown to be correlated with plasma and brain variables. Milk contains many components like MFGM and oligosaccharides, which are increasingly recognised for their potential role in early postnatal development. However, this study did not examine their profiles in bovine, caprine, and ovine milk, and their implication for early postnatal development.

Using an untargeted LC-MS approach provided broad coverage of brain and blood metabolomes. However, this approach does not identify other low molecular weight volatile compounds that gas chromatography-mass spectrometry could. In addition, LC-MS/MS is better than LC-MS for identifying unknown compounds; it provides more fragment information to help unknown compounds identification.

Finally, using NanoString technology allowed the detection of true mRNA abundance, making the gene expression profiling accurate compared to other methods like RNA

sequencing. However, NanoString requires prior knowledge of the known sequence for designing probes, limiting the number of genes for the analysis.

## 7.3 Future directions

Future research is required to understand whether the observed changes in brain metabolite and gene levels in response to milk treatments are sufficient to bring about changes in brain function (assessed through imaging techniques like functional magnetic resonance imaging) and behaviour (evaluated through behavioural tasks like objection recognition test) associated with cognitive development. Further studies based on standardised milk intakes are essential to investigate if the changes observed in plasma and brain variables in response to different milk under the current study conditions were affected by milk volumes.

Longitudinal piglet studies and multiple sampling points are required to identify biomarkers that can be targeted to optimise the cognitive outcomes in infants. These studies combined with stable isotope experiments should be performed in future to understand the possible incorporation of dietary lipids into brain tissue and blood plasma lipid kinetics. Future longitudinal research is essential on the impact of different ruminant milk sources on early postnatal shaping of the gut microbiota and neurodevelopment via microbiota-gut-brain axis mediators (e.g., neural mediators) to possibly optimise brain development in this sensitive period.

Future analyses on gut tissues involving metabolomics profiling, morphometry, permeability, and targeted gene and protein expression profiling would provide insights into gut-mediated dietary effects on brain development in the early postnatal period by linking the gut responses to the milk treatments to plasma and brain metabolite changes.

Research in human infants (e.g., metabolomics screening of neonatal dried blood spot samples) needs to be conducted in future to understand whether milk from different ruminant species could influence cognitive development in infants.

Furthermore, future studies involving different ruminant milk-based infant formulas available in the market are essential to understand whether specific ruminants' milk components added to the formulas could influence early postnatal cognitive development.

## 7.4 Thesis conclusion

Overall, this thesis showed that consumption of milk of different ruminant species in the early postnatal period influenced the peripheral blood plasma metabolome and metabolome and gene expression of the areas of the brain crucial for cognitive development in piglets as a model for human infants. In addition, this thesis suggested lipids as potential metabolites in the context of ruminants' milk influencing brain development in the early postnatal period. Furthermore, this thesis highlighted the brain region-dependent ruminant milk effect, suggesting the importance of studying individual brain regions in the context of early-life nutrition. The new knowledge gained through this thesis lays the foundation for future studies on the effects of milk formula on an infant's brain function and behaviour.

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# Appendices

## Appendix tables

**Table A1 Body weight gain data of piglets from experimental day 1 until day 15**

| Pig id | Milk treatment group | Day 1 BW | Day 3 BW | Day 5 BW | Day 7 BW | Day 13 BW | Day 15 BW |
|---|---|---|---|---|---|---|---|
| 3 | Bovine | 3.3 | 3.7 | 3.7 | 3.9 | 5 | 5 |
| 6 | Bovine | 2.7 | 2.8 | 3.15 | 3.4 | 4.4 | 4.4 |
| 7 | Ovine | 2.4 | 2.5 | 2.7 | 3 | 3.9 | 3.9 |
| 14 | Caprine | 2.8 | 2.7 | 2.9 | 3.2 | 4.2 | 4.2 |
| 23 | Caprine | 3.4 | 3.2 | 3.69 | 4 | 5.3 | 5.3 |
| 25 | Ovine | 3.4 | 3.2 | 3.53 | 3.7 | 4.7 | 4.7 |
| 42 | Ovine | 3.5 | 3.6 | 4.1 | 4.4 | 5.6 | 5.6 |
| 48 | Ovine | 3.04 | 3.11 | 3.4 | 3.8 | 4.8 | 5.2 |
| 49 | Caprine | 2.37 | 2.4 | 2.65 | 3 | 3.9 | 4.2 |
| 50 | Bovine | 2.67 | 2.6 | 2.89 | 3.3 | 4.3 | 4.7 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 53 | Bovine | 2.68 | 2.7 | 2.9 | 3.3 | 4.2 | 4.2 |
| 55 | Caprine | 3.35 | 3.5 | 3.8 | 4.2 | 5.5 | 5.5 |
| 63 | Caprine | 3.05 | 3.22 | 3.51 | 4.1 | 5.2 | 5.2 |
| 64 | Bovine | 3.51 | 3.73 | 4.1 | 4.6 | 6 | 6 |
| 65 | Ovine | 2.84 | 2.92 | 3.18 | 3.5 | 4.6 | 4.6 |
| 68 | Ovine | 2.52 | 2.76 | 3.04 | 3.4 | 4.8 | 5 |
| 72 | Caprine | 2.9 | 3.06 | 3.2 | 3.8 | 4.8 | 5.1 |
| 73 | Bovine | 1.96 | 2 | 2.24 | 2.5 | 3.4 | 3.8 |
| 75 | Caprine | 3.06 | 3.21 | 3.6 | 4.1 | 5.3 | 5.3 |
| 76 | Bovine | 3.16 | 3.29 | 3.64 | 4.1 | 5.1 | 5.1 |
| 78 | Caprine | 3.25 | 3.38 | 3.6 | 4.1 | 5.2 | 5.8 |
| 80 | Ovine | 3.4 | 3.25 | 3.6 | 4 | 5.3 | 5.3 |
| 82 | Bovine | 2.96 | 2.88 | 3.1 | 3.4 | 4.2 | 4.8 |
| 83 | Ovine | 2.9 | 3.15 | 3.49 | 3.9 | 5 | 5.4 |

**Table A2 Lipids with a significant difference in relative intensities between the bovine (n=3), caprine (n=3), and ovine (n=3) milk samples**[+]

| Lipid | FDR | Log2fold change | | |
|---|---|---|---|---|
| | | Ovine vs Bovine | Ovine vs Caprine | Caprine vs Bovine |
| TG 8:0_12:0_14:0 | 0.01 | 0.61 | 1.1* | -0.49 |
| TG 9:0_9:0_16:0 | 0.01 | 0.59 | 1.09* | -0.49 |
| TG 10:0_14:0_16:0 | 0.01 | 0.7 | 0.65 | 0.05 |
| TG 12:0_12:0_16:0 | 0.01 | 0.74 | 0.76 | -0.02 |
| TG 12:0_14:0_16:0 | 0.02 | 0.67 | 0.58 | 0.09 |
| TG 13:0_15:0_19:1 | 0.00 | 0.66 | 1.18* | -0.52 |
| TG 14:0_14:0_14:0 | 0.04 | 0.68 | 0.49 | 0.19 |
| TG 14:0_16:0_18:2 | 0.01 | 0.37 | 0.82 | -0.45 |
| TG 14:0_18:1_18:1 | 0.01 | 0.33 | 0.71 | -0.37 |
| TG 16:0_16:0_16:2 | 0.01 | 0.49 | 0.83 | -0.34 |
| TG 16:0_16:0_18:2 | 0.02 | 0.33 | 0.7 | -0.36 |
| TG 16:0_16:0_20:3 | 0.02 | 0.76 | 0.8 | -0.04 |
| TG 16:0_18:1_18:2 | 0.01 | 0.92 | 0.9 | 0.02 |

| | | | | |
|---|---|---|---|---|
| TG 26:0 | 0.01 | 1.99* | 1.99* | 0.01 |
| TG 28:0 | 0.01 | 1.62* | 1.92* | -0.3 |
| TG 30:0 | 0.01 | 1.43* | 1.09* | 0.34 |
| TG 32:0 | 0.01 | 0.8 | 1.34* | -0.53 |
| TG 32:1 | 0.03 | 1.12 | 0.5 | 0.62 |
| TG 33:0 | 0.03 | 0.81 | 1.6* | -0.78 |
| TG 35:0 | 0.01 | 0.65 | 1.31* | -0.66 |
| TG 36:0 | 0.01 | 0.27 | 0.83 | -0.56 |
| TG 36:1 | 0.00 | 0.48 | 1.06* | -0.59 |
| TG 37:0 | 0.00 | 0.65 | 1.17* | -0.53 |
| TG 37:1 | 0.00 | 0.89 | 1.46* | -0.56 |
| TG 38:0 | 0.01 | 0.45 | 0.81 | -0.36 |
| TG 38:1 | 0.00 | 0.52 | 0.94 | -0.41 |
| TG 38:2 | 0.00 | 1.03* | 1.22* | -0.18 |
| TG 38:3 | 0.00 | 1.57* | 2.59* | -1.02 |
| TG 39:0 | 0.01 | 0.97 | 1.16* | -0.2 |

| | | | | |
|---|---|---|---|---|
| TG 39:1 | 0.00 | 0.92 | 1.25* | -0.33 |
| TG 39:2 | 0.00 | 1.79* | 2.04* | -0.25 |
| TG 40:1 | 0.01 | 0.71 | 0.9 | -0.19 |
| TG 41:0 | 0.01 | 0.98 | 0.98 | 0 |
| TG 41:1 | 0.03 | 1.04* | 0.86 | 0.18 |
| TG 41:2 | 0.00 | 2.57* | 1.74* | 0.83 |
| TG 42:1 | 0.01 | 0.89 | 0.62 | 0.27 |
| TG 42:2 | 0.00 | 0.97 | 0.52 | 0.45 |
| TG 42:3 | 0.00 | 1.36* | 0.92 | 0.44 |
| TG 43:0 | 0.01 | 0.83 | 0.96 | -0.12 |
| TG 43:1 | 0.01 | 1.36* | 1.4* | -0.04 |
| TG 43:2 | 0.02 | 1.63* | 1.81* | -0.18 |
| TG 44:0 | 0.03 | 0.4 | 0.57 | -0.16 |
| TG 44:1 | 0.01 | 0.89 | 0.51 | 0.39 |
| TG 44:2 | 0.00 | 1.22* | 0.49 | 0.72 |
| TG 44:3 | 0.01 | 1.32* | 0.8 | 0.52 |

| | | | | |
|---|---|---|---|---|
| TG 45:0 | 0.02 | 0.41 | 1.06* | -0.65 |
| TG 45:1 | 0.01 | 0.8 | 0.98 | -0.18 |
| TG 46:0 | 0.00 | 0.18 | 0.75 | -0.57 |
| TG 46:1 | 0.01 | 0.38 | 0.64 | -0.26 |
| TG 46:2 | 0.01 | 0.88 | 0.45 | 0.43 |
| TG 46:3 | 0.00 | 1.39* | 0.52 | 0.86 |
| TG 47:0 | 0.00 | 0.33 | 1.08* | -0.74 |
| TG 48:0 | 0.01 | 0.13 | 0.71 | -0.59 |
| TG 48:1 | 0.00 | 0.26 | 0.88 | -0.62 |
| TG 48:3 | 0.01 | 0.56 | 0.95 | -0.4 |
| TG 49:0 | 0.00 | 0.44 | 0.99 | -0.55 |
| TG 49:1 | 0.00 | 0.54 | 1.25* | -0.71 |
| TG 49:2 | 0.01 | 0.63 | 1.12* | -0.48 |
| TG 49:3 | 0.01 | 1.71* | 1.63* | 0.09 |
| TG 50:0 | 0.03 | 0.12 | 0.46 | -0.33 |
| TG 50:1 | 0.01 | 0.1 | 0.69 | -0.6 |

| | | | | |
|---|---|---|---|---|
| TG 50:3 | 0.01 | 0.73 | 1.12* | -0.39 |
| TG 50:4 | 0.02 | 0.91 | 1.28* | -0.37 |
| TG 51:0 | 0.01 | 0.72 | 1.06* | -0.35 |
| TG 51:1 | 0.00 | 0.62 | 1.02* | -0.41 |
| TG 51:2 | 0.00 | 0.94 | 1.2* | -0.26 |
| TG 51:3 | 0.01 | 1.21* | 1.29* | -0.09 |
| TG 52:1 | 0.01 | 0.44 | 0.65 | -0.21 |
| TG 52:2 | 0.01 | 0.58 | 0.78 | -0.2 |
| TG 52:4 | 0.01 | 1.15 | 1.37* | -0.21 |
| TG 52:5 | 0.01 | 1.49 | 2.39* | -0.9 |
| TG 53:2 | 0.00 | 0.81 | 0.86 | -0.04 |
| TG 53:3 | 0.02 | 1.24 | 1.27* | -0.03 |
| TG 53:4 | 0.01 | 1.83* | 1.53* | 0.3 |
| TG 54:0 | 0.04 | 0.82 | 0.85 | -0.03 |
| TG 54:1 | 0.00 | 0.93 | 0.85 | 0.08 |
| TG 54:2 | 0.00 | 1.03* | 0.81 | 0.22 |

| | | | | |
|---|---|---|---|---|
| TG 54:3 | 0.01 | 1.04* | 0.7 | 0.33 |
| TG 54:4 | 0.02 | 1.23* | 0.85 | 0.38 |
| TG 54:5 | 0.01 | 1.26* | 0.99 | 0.27 |
| TG 56:6 | 0.00 | 2.66* | 2.62* | 0.04 |

*+VIP value >1 from the PLS-DA model was used to select the major contributing lipids. One-way ANOVA was used to determine the significant lipid relative intensities between milk types (FDR < 0.05). Only the polar metabolites that satisfied the multivariate and ANOVA analysis criteria are shown. *Lipids with significant log2 fold change > ± 1 (FDR < 0.05 using t-test) between milk. VIP, variable importance in projection; FDR, false discovery rate; TG, triglyceride*

**Table A3 Lipid composition of bovine, caprine, and ovine raw whole milk, and lipid provided per day**

| Lipid$^{+}$ | Raw milk (in %) | | | Intake*(in mg)$^{+}$ | | |
|---|---|---|---|---|---|---|
| | Bovine | Caprine | Ovine | Bovine | Caprine | Ovine |
| TG 8:0_12:0_14:0 | 3.00 | 2.51 | 3.05 | 1129.84±198.05$^{b}$ | 966.47±123.21$^{b}$ | 1508.16±172.28$^{a}$ |
| TG 9:0_9:0_16:0 | 1.66 | 1.39 | 1.67 | 626.43±109.81$^{b}$ | 536.28±68.37$^{b}$ | 826.95±94.47$^{a}$ |
| TG 10:0_14:0_16:0 | 2.34 | 2.86 | 2.52 | 880.75±154.39$^{b}$ | 1100.07±140.24$^{a}$ | 1250.49±142.85$^{a}$ |
| TG 12:0_12:0_16:0 | 1.26 | 1.47 | 1.40 | 475.64±83.38$^{c}$ | 563.84±71.88$^{b}$ | 693.11±79.18$^{a}$ |
| TG 12:0_14:0_16:0 | 2.18 | 2.74 | 2.31 | 823.54±144.36$^{b}$ | 1054.04±134.37$^{a}$ | 1143.41±130.62$^{a}$ |
| TG 13:0_15:0_19:1 | 0.62 | 0.51 | 0.65 | 233.38±40.91$^{b}$ | 195.72±24.95$^{b}$ | 322.84±36.88$^{a}$ |
| TG 14:0_14:0_14:0 | 0.90 | 1.21 | 0.96 | 337.8±59.21$^{b}$ | 463.87±59.14$^{a}$ | 473.15±54.05$^{a}$ |
| TG 14:0_16:0_18:2 | 1.99 | 1.71 | 1.71 | 748.8±131.26$^{ab}$ | 658.38±83.93$^{b}$ | 845.81±96.62$^{a}$ |
| TG 14:0_18:1_18:1 | 4.32 | 3.94 | 3.63 | 1629.22±285.59 | 1513.65±192.97 | 1798.32±205.43 |
| TG 16:0_16:0_16:2 | 0.46 | 0.43 | 0.43 | 173.45±30.41$^{b}$ | 165.31±21.07$^{b}$ | 213.19±24.35$^{a}$ |
| TG 16:0_16:0_18:2 | 0.60 | 0.55 | 0.50 | 226.36±39.68 | 211.68±26.99 | 249.28±28.48 |
| TG 16:0_16:0_20:3 | 0.36 | 0.42 | 0.41 | 136.73±23.97$^{b}$ | 160.39±20.45$^{b}$ | 202.96±23.18$^{a}$ |
| TG 16:0_18:1_18:2 | 2.17 | 2.59 | 2.73 | 816.73±143.17$^{c}$ | 994.09±126.73$^{b}$ | 1350.06±154.22$^{a}$ |
| TG 26:0 | 0.06 | 0.07 | 0.15 | 21.6±3.78$^{b}$ | 26.15±3.33$^{b}$ | 75.29±8.6$^{a}$ |
| TG 28:0 | 0.16 | 0.15 | 0.33 | 59.93±10.51$^{b}$ | 58.65±7.48$^{b}$ | 161.11±18.4$^{a}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 30:0 | 0.21 | 0.31 | 0.38 | 79.19±13.88$^{c}$ | 120.7±15.39$^{b}$ | 187.24±21.39$^{a}$ |
| TG 32:0 | 0.43 | 0.35 | 0.50 | 160.5±28.13$^{b}$ | 133.57±17.03$^{b}$ | 245.19±28.01$^{a}$ |
| TG 32:1 | 0.11 | 0.20 | 0.16 | 40.81±7.15$^{b}$ | 75.65±9.64$^{a}$ | 77.61±8.87$^{a}$ |
| TG 33:0 | 0.14 | 0.09 | 0.16 | 52.14±9.14$^{b}$ | 36.51±4.66$^{c}$ | 80.2±9.16$^{a}$ |
| TG 35:0 | 0.61 | 0.45 | 0.63 | 228.48±40.05$^{b}$ | 173.93±22.17$^{c}$ | 313.28±35.79$^{a}$ |
| TG 36:0 | 2.84 | 2.28 | 2.29 | 1072.8±188.06$^{a}$ | 876.73±111.77$^{b}$ | 1135.21±129.68$^{a}$ |
| TG 36:1 | 2.12 | 1.66 | 1.96 | 797.68±139.83$^{b}$ | 639.44±81.52$^{c}$ | 971.21±110.94$^{a}$ |
| TG 37:0 | 0.64 | 0.53 | 0.67 | 242.72±42.55$^{b}$ | 202.8±25.85$^{c}$ | 332.29±37.96$^{a}$ |
| TG 37:1 | 0.36 | 0.29 | 0.45 | 137.17±24.04$^{b}$ | 111.72±14.24$^{c}$ | 222.7±25.44$^{a}$ |
| TG 38:0 | 3.59 | 3.30 | 3.27 | 1352.55±237.09$^{b}$ | 1269.05±161.78$^{b}$ | 1620.37±185.1$^{a}$ |
| TG 38:1 | 2.05 | 1.82 | 1.96 | 771.73±135.28$^{b}$ | 698.02±88.99$^{b}$ | 970.54±110.87$^{a}$ |
| TG 38:2 | 0.89 | 0.92 | 1.21 | 333.98±58.55$^{b}$ | 354.59±45.21$^{b}$ | 598.43±68.36$^{a}$ |
| TG 38:3 | 0.11 | 0.06 | 0.22 | 41.08±7.2$^{b}$ | 24.43±3.11$^{c}$ | 106.92±12.22$^{a}$ |
| TG 39:0 | 0.37 | 0.39 | 0.49 | 141.36±24.78$^{b}$ | 148.63±18.95$^{b}$ | 241.78±27.62$^{a}$ |
| TG 39:1 | 0.38 | 0.36 | 0.48 | 144.35±25.3$^{b}$ | 138.12±17.61$^{b}$ | 238.24±27.21$^{a}$ |
| TG 39:2 | 0.08 | 0.08 | 0.19 | 30.94±5.42$^{b}$ | 31.43±4.01$^{b}$ | 93.61±10.69$^{a}$ |
| TG 40:1 | 2.78 | 2.87 | 3.02 | 1047.74±183.66$^{b}$ | 1104.87±140.86$^{b}$ | 1497.39±171.05$^{a}$ |
| TG 41:0 | 0.32 | 0.38 | 0.43 | 122.17±21.42$^{c}$ | 147.43±18.79$^{b}$ | 211.29±24.14$^{a}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 41:1 | 0.21 | 0.28 | 0.29 | 80.28±14.07$^{c}$ | 109.48±13.96$^{b}$ | 144.02±16.45$^{a}$ |
| TG 41:2 | 0.03 | 0.07 | 0.14 | 13.05±2.29$^{c}$ | 27.85±3.55$^{b}$ | 67.68±7.73$^{a}$ |
| TG 42:1 | 1.65 | 2.35 | 2.04 | 622.43±109.11$^{b}$ | 904.13±115.26$^{a}$ | 1012.19±115.63$^{a}$ |
| TG 42:2 | 0.71 | 1.14 | 0.92 | 266±46.63$^{b}$ | 437.38±55.76$^{a}$ | 455.14±51.99$^{a}$ |
| TG 42:3 | 0.23 | 0.37 | 0.39 | 86.67±15.19$^{c}$ | 141.85±18.08$^{b}$ | 194.69±22.24$^{a}$ |
| TG 43:0 | 0.36 | 0.39 | 0.43 | 137.11±24.03$^{b}$ | 151.42±19.3$^{b}$ | 213.93±24.44$^{a}$ |
| TG 43:1 | 0.23 | 0.26 | 0.39 | 85.46±14.98$^{b}$ | 99.89±12.73$^{b}$ | 192.09±21.94$^{a}$ |
| TG 43:2 | 0.05 | 0.05 | 0.10 | 17.45±3.06$^{b}$ | 18.58±2.37$^{b}$ | 47.44±5.42$^{a}$ |
| TG 44:0 | 2.53 | 2.66 | 2.22 | 953.37±167.12 | 1024.01±130.54 | 1101.26±125.8 |
| TG 44:1 | 2.01 | 3.10 | 2.49 | 756.52±132.61$^{b}$ | 1190.51±151.77$^{a}$ | 1230.88±140.61$^{a}$ |
| TG 44:2 | 0.62 | 1.21 | 0.96 | 233.52±40.93$^{b}$ | 464.31±59.19$^{a}$ | 475.4±54.31$^{a}$ |
| TG 44:3 | 0.20 | 0.34 | 0.34 | 75.96±13.31$^{c}$ | 130.99±16.7$^{b}$ | 166.13±18.98$^{a}$ |
| TG 45:0 | 0.60 | 0.45 | 0.53 | 224.42±39.34$^{b}$ | 171.95±21.92$^{c}$ | 260.6±29.77$^{a}$ |
| TG 45:1 | 0.32 | 0.33 | 0.37 | 120.32±21.09$^{b}$ | 127.99±16.31$^{b}$ | 183.41±20.95$^{a}$ |
| TG 46:0 | 3.21 | 2.55 | 2.42 | 1209.98±212.1$^{a}$ | 980.71±125.03$^{b}$ | 1197.4±136.78$^{a}$ |
| TG 46:1 | 3.24 | 3.20 | 2.81 | 1222.56±214.31 | 1229.35±156.72 | 1389.52±158.73 |
| TG 46:2 | 0.94 | 1.50 | 1.16 | 354.22±62.09$^{b}$ | 575.09±73.31$^{a}$ | 572.36±65.38$^{a}$ |
| TG 46:3 | 0.24 | 0.51 | 0.41 | 88.95±15.59$^{b}$ | 194.7±24.82$^{a}$ | 203.54±23.25$^{a}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 47:0 | 0.78 | 0.55 | 0.65 | 293.58±51.46[a] | 211.03±26.9[b] | 323.96±37.01[a] |
| TG 48:0 | 2.75 | 2.17 | 2.00 | 1037.66±181.9[a] | 832.89±106.18[b] | 991.28±113.24[a] |
| TG 48:1 | 6.11 | 4.71 | 4.89 | 2303.56±403.8[a] | 1810.54±230.82[b] | 2422.15±276.69[a] |
| TG 48:3 | 0.41 | 0.37 | 0.40 | 154.55±27.09[b] | 141.28±18.01[b] | 198.85±22.72[a] |
| TG 49:0 | 0.58 | 0.47 | 0.52 | 217.85±38.19[b] | 179.39±22.87[c] | 259.37±29.63[a] |
| TG 49:1 | 1.32 | 0.95 | 1.28 | 496.07±86.96[b] | 365.8±46.63[c] | 632.65±72.27[a] |
| TG 49:2 | 0.43 | 0.36 | 0.45 | 22.38±3.92[c] | 28.59±3.64[b] | 64.15±7.33[a] |
| TG 49:3 | 0.06 | 0.07 | 0.13 | 723.17±126.77 | 690.57±88.04 | 689.43±78.76 |
| TG 50:0 | 1.92 | 1.80 | 1.39 | 2899.11±508.2[a] | 2311.08±294.63[b] | 2713.22±309.94[ab] |
| TG 50:1 | 7.69 | 6.01 | 5.48 | 400.8±70.26[b] | 367.68±46.87[b] | 581.85±66.47[a] |
| TG 50:3 | 1.06 | 0.96 | 1.17 | 75.35±13.21[b] | 70.03±8.93[b] | 124.04±14.17[a] |
| TG 50:4 | 0.20 | 0.18 | 0.25 | 108.23±18.97[b] | 102.2±13.03[b] | 155.62±17.78[a] |
| TG 51:0 | 0.29 | 0.27 | 0.31 | 374.57±65.66[b] | 340.46±43.4[b] | 502.62±57.42[a] |
| TG 51:1 | 0.99 | 0.89 | 1.01 | 278.63±48.84[b] | 279.26±35.6[b] | 466.54±53.29[a] |
| TG 51:2 | 0.74 | 0.73 | 0.94 | 75.94±13.31[b] | 86.18±10.99[b] | 153.66±17.55[a] |
| TG 51:3 | 0.20 | 0.22 | 0.31 | 1346.44±236.02 | 1401.99±178.73 | 1601.27±182.92 |
| TG 52:1 | 3.57 | 3.65 | 3.23 | 2123.3±372.2[b] | 2228.1±284.05[b] | 2771.13±316.56[a] |
| TG 52:2 | 5.63 | 5.79 | 5.60 | 251.4±44.07[b] | 260.86±33.26[b] | 488.93±55.85[a] |

| | | | | | | |
|---|---|---|---|---|---|---|
| TG 52:4 | 0.67 | 0.68 | 0.99 | 40.18±7.04[b] | 25.88±3.3[c] | 98.61±11.27[a] |
| TG 52:5 | 0.11 | 0.07 | 0.20 | 153.25±26.87[b] | 179.26±22.85[b] | 235.83±26.94[a] |
| TG 53:2 | 0.41 | 0.47 | 0.48 | 15.61±2.74[c] | 23.1±2.95[b] | 48.52±5.54[a] |
| TG 53:3 | 0.18 | 0.21 | 0.29 | 68.8±12.06[b] | 81.35±10.37[b] | 142.51±16.28[a] |
| TG 53:4 | 0.04 | 0.06 | 0.10 | 15.61±2.74[c] | 23.1±2.95[b] | 48.52±5.54[a] |
| TG 54:0 | 0.12 | 0.14 | 0.14 | 45.16±7.91[b] | 53.18±6.78[b] | 69.82±7.98[a] |
| TG 54:1 | 0.64 | 0.80 | 0.81 | 241.15±42.27[c] | 307.64±39.22[b] | 403.19±46.06[a] |
| TG 54:2 | 1.47 | 2.02 | 2.00 | 554.97±97.28[c] | 776.39±98.98[b] | 992.07±113.33[a] |
| TG 54:3 | 1.69 | 2.51 | 2.31 | 636.84±111.63[c] | 966.68±123.24[b] | 1143.11±130.59[a] |
| TG 54:4 | 0.81 | 1.24 | 1.27 | 305.23±53.51[c] | 478.4±60.99[b] | 628.47±71.79[a] |
| TG 54:5 | 0.33 | 0.46 | 0.52 | 123.43±21.64[c] | 178.7±22.78[b] | 258.22±29.5[a] |
| TG 56:6 | 0.03 | 0.04 | 0.14 | 12.21±2.14[b] | 15.09±1.92[b] | 67.38±7.7[a] |

[+]The percentage of each lipid was calculated within the lipid class. **Amount ingested per day average calculated from ~postnatal day 10 onwards (as first three days of the study (~postnatal day 7 to 9), there was a significant amount of spill and refusals by the pigs, and the intake for these days were not included in the table. Intake is represented as the amount of each TG in the total amount of fat consumed per day on average. Data analysed via one-way ANOVA with post hoc Fisher's least significant difference test and values are represented as mean ± standard deviation. [a-c] Values in the same row without a common superscript are different (FDR <0.05) from each other.* TG, Triglyceride; DG, diglyceride

**Table A4 Amino acid composition of bovine, caprine, and ovine raw whole milk, and amino acid provided per day[+]**

| Amino acid | Raw milk** (mg/mL) | | | Intake[++] (mg) | | |
|---|---|---|---|---|---|---|
| | Bovine | Caprine | Ovine | Bovine | Caprine | Ovine |
| Alanine | 1.10±0.01[b] | 0.90±0.05[c] | 2.13±0.03[a] | 1147.17±201.09[b] | 1091.34±139.13[b] | 1679.32±191.84[a] |
| Arginine | 1.16±0.04[b] | 0.87±0.05[c] | 2.04±0.07[a] | 1206.12±211.43[b] | 1049.59±133.81[b] | 1608.97±183.80[a] |
| Aspartic acid | 2.54±0.08[b] | 2.15±0.04[c] | 4.56±0.03[a] | 2650.97±464.70[b] | 2610.88±332.85[b] | 3596.19±410.81[a] |
| Cysteine | 0.21±0.00[b] | 0.20±0.01[b] | 0.39±0.02[a] | 222.09±38.93[b] | 246.92±31.48[b] | 310.56±35.480[a] |
| Glutamic acid | 6.88±0.08[b] | 5.93±0.09[c] | 11.47±0.26[a] | 7166.53±1256.25[b] | 7191.89±916.86[b] | 9047.56±1033.55[a] |
| Glycine | 0.70±0.00[b] | 0.55±0.02[c] | 1.21±0.01[a] | 732.76±128.45[b] | 673.18±85.82[b] | 955.55±109.16[a] |
| Histidine | 0.85±0.01[b] | 0.75±0.02[c] | 1.48±0.02[a] | 890.37±156.08[b] | 911.77±116.24[b] | 1167.14±133.33[a] |
| Isoleucine | 1.69±0.04[b] | 1.39±0.04[c] | 2.74±0.01[a] | 1760.16±308.55[b] | 1690.24±215.48[b] | 2160.55±246.81[a] |
| Leucine | 3.20±0.09[b] | 2.75±0.03[c] | 5.53±0.13[a] | 3331.15±583.93[b] | 3338.34±425.59[b] | 4363.31±498.44[a] |
| Lysine | 2.72±0.03[b] | 2.36±0.02[c] | 4.65±0.13[a] | 2829.36±495.97[b] | 2859.78±364.58[b] | 3665.56±418.73[a] |
| Methionine | 0.81±0.00[b] | 0.64±0.01[c] | 1.44±0.05[a] | 922.32±161.68[b] | 892.54±113.78[b] | 1259.72±143.9[a] |
| Phenylalanine | 1.57±0.04[b] | 1.40±0.01[c] | 2.61±0.04[a] | 1631±285.91[b] | 1698.11±216.48[b] | 2057.5±235.04[a] |
| Proline | 3.31±0.05[b] | 3.11±0.08[b] | 5.91±0.11[a] | 3451.23±604.98[b] | 3767.88±480.35[b] | 4658.35±532.14[a] |
| Serine | 1.74±0.03[b] | 1.43±0.03[c] | 2.82±0.07[a] | 1811.94±317.62[b] | 1733.19±220.96[b] | 2222.91±253.93[a] |
| Threonine | 1.54±0.06[b] | 1.53±0.05[b] | 2.38±0.05[a] | 1603.26±281.04 | 1858.13±236.88 | 1874.07±214.08 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Tryptophan | 0.47±0.01[b] | 0.40±0.01[c] | 0.83±0.01[a] | 487.65±85.48[b] | 489.79±62.44[b] | 656.78±75.03[a] |
| Tyrosine | 1.68±0.03[b] | 1.16±0.03[c] | 2.67±0.05[a] | 1748.62±306.52[b] | 1401.2±178.63[c] | 2104.07±240.36[a] |
| Valine | 2.17±0.05[b] | 2.09±0.05[b] | 3.69±0.07[a] | 2257.67±395.76[b] | 2529.62±322.49[b] | 2912.04±332.66[a] |

*[+]Data was analysed via one-way ANOVA with post hoc Fisher's least significant difference test. **Values are represented as mean ± standard error of the mean of three batches of each milk type used during the pig study. [a-c] Values in the same row without a common superscript are different (FDR <0.05) from each other. [++]Amount ingested per day average calculated from ~postnatal day 10 onwards (as first three days of the study ( ~postnatal day 7 to 9), there was a significant amount of spill and refusals by the pigs, and the intake for these days are not included in the table) BW, body weight.*

**Table A5 Milk composition of bovine, caprine, and ovine raw whole milk, and amounts provided per day***

| Component** | Raw milk (g/100ml) | | | Intake (g) | | |
|---|---|---|---|---|---|---|
| | Bovine | Caprine | Ovine | Bovine | Caprine | Ovine |
| Dry matter | 13.10± 0.20$^{b}$ | 11.23 ± 0.31$^{c}$ | 17.6 0± 0.10$^{a}$ | 136.48±8.46 | 136.26±6.57 | 138.81±5.61 |
| Protein | 3.61 ± 0.07$^{b}$ | 3.16 ± 0.11$^{c}$ | 6.27 ± 0.08$^{a}$ | 37.71±2.34$^{b}$ | 38.45±1.85$^{b}$ | 49.53±2$^{a}$ |
| Fat | 4.06 ± 0.27$^{b}$ | 3.22 ± 0.17$^{c}$ | 6.31 ± 0.09$^{a}$ | 42.3±2.62$^{b}$ | 39.18±1.89$^{b}$ | 49.76±2.01$^{a}$ |
| Lactose | 4.56 ± 0.09$^{a}$ | 3.91 ± 0.07$^{c}$ | 4.16 ± 0.06$^{b}$ | 47.51±2.94$^{a}$ | 47.55±2.29$^{a}$ | 32.89±1.33$^{b}$ |
| Gross energy | 75.80 ± 1.84$^{b}$ | 61.11 ± 1.23$^{c}$ | 107.50 ± 0.27$^{a}$ | 789.83±48.95 | 741.26±35.72 | 847.82±34.24 |

**Data analysed via one-way ANOVA with post hoc Fisher's least significant difference test. **Values are represented as mean ± standard deviation of three batches of each milk type and nutrient intake of piglets. $^{a-c}$ Values in the same row without a common superscript are different (FDR <0.05) from each other*

**Table A6 Correlations between the first components of two data sets and weights of design matrix selected for the DIABLO models**

| Data sets | Brain tissue type | PLS correlation value | Design matrix value** |
|---|---|---|---|
| Plasma lipids and brain lipids | Hippocampus | 0.6 | 0.6 |
| | Prefrontal cortex | 0.5 | 0.6 |
| | Striatum | 0.8 | 1 |

**Correlated values of the first component from each PLS model are represented. **Design matrix value ranges between 0 and 1 and is set manually based on the correlation value obtained in the PLS model.*

**Table A7 Number of components and features used for final DIABLO models***

| Data sets | Brain tissue type | No. of components | No. of features | |
|---|---|---|---|---|
| | | | Plasma lipid | Brain lipid |
| Plasma lipids and brain lipids | Hippocampus | 1 | 5 | 8 |
| | | 2 | 4 | 3 |
| | Prefrontal cortex | 1 | 5 | 6 |
| | | 2 | 5 | 5 |
| | Striatum | 1 | 5 | 6 |
| | | 2 | 5 | 5 |

** The number of components was selected based on the lowest balanced error rate and centroid distance across the five components used in the initial model, and the number of features was manually tuned and selected.*

Appendix figures

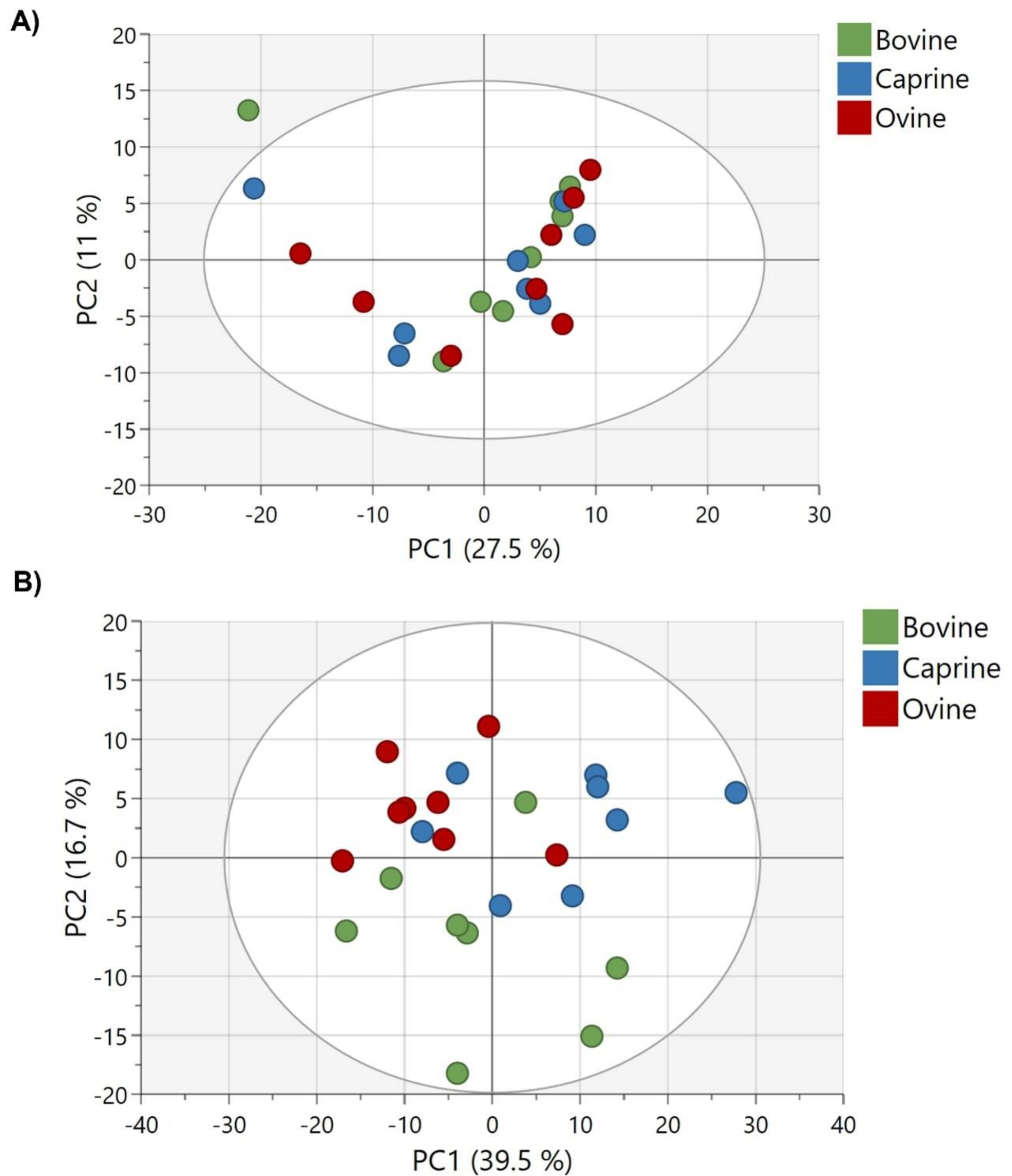



**Figure A1 Principal component analysis (PCA) score plot showing A) polar B) non-polar (lipids) metabolites differences in plasma of piglets following bovine, caprine, or ovine milk treatments.**

The first two principal components (PC) are plotted. Percentages of variation explained by each principal component are indicated along the axes. PC, principal component

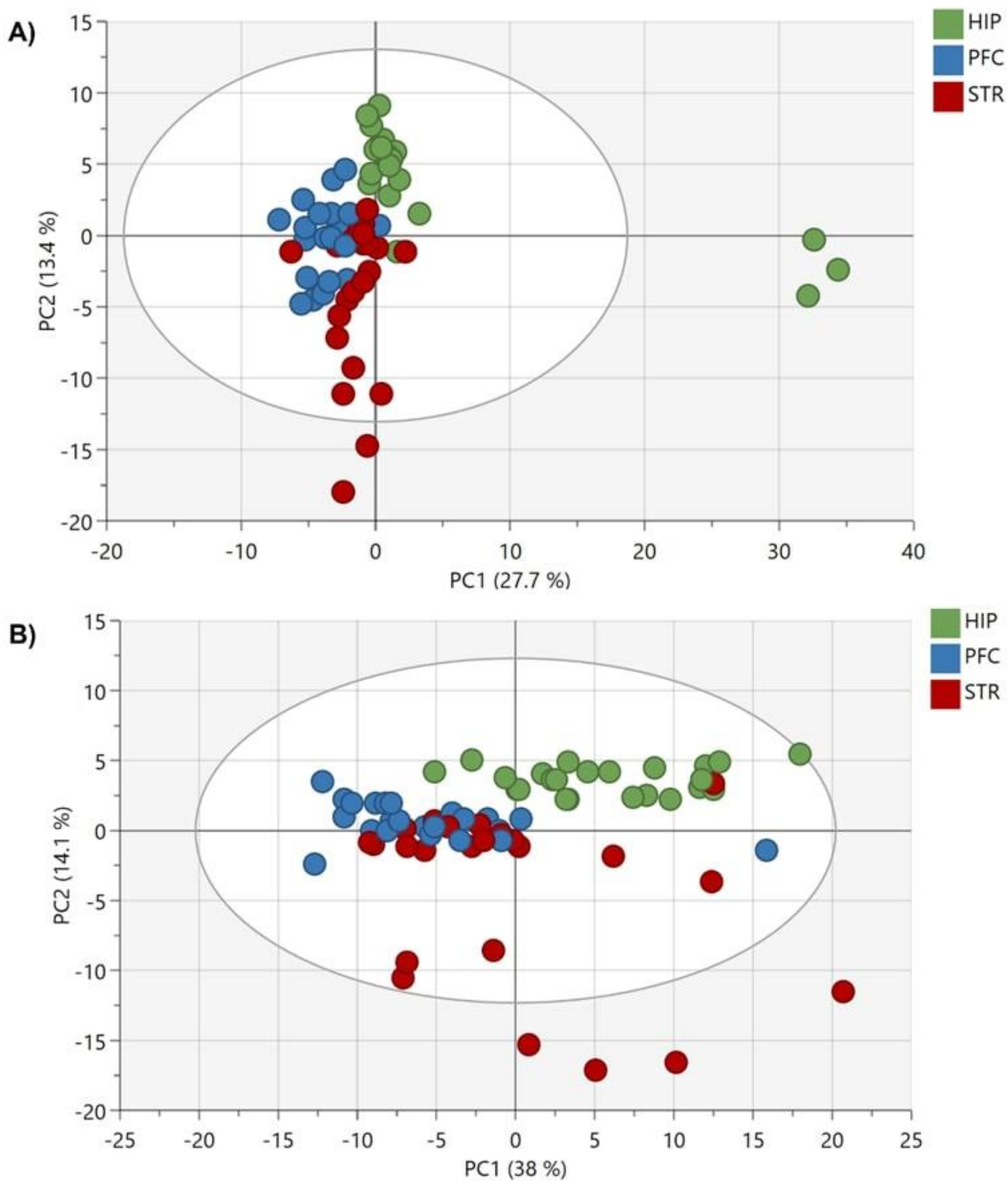



**Figure A2 Principal component analysis (PCA) score plot showing A) polar metabolites and B) non-polar (lipid) metabolites difference between hippocampal (HIP), prefrontal cortex (PFC), and striatal (STR) tissues of piglets exclusively fed bovine, caprine, or ovine milk for 15 days.**

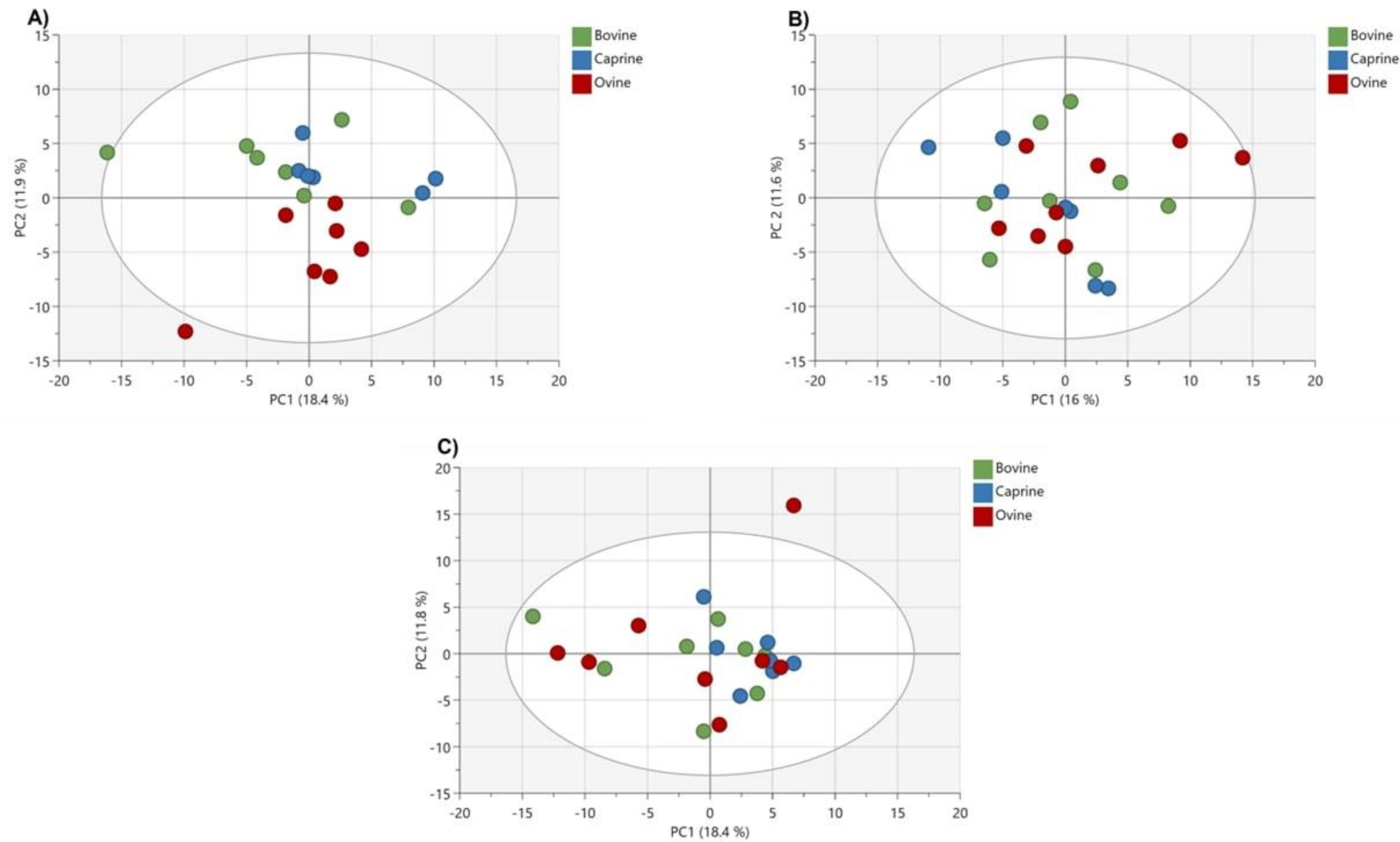



**Figure A3 Principal component analysis (PCA) showing polar metabolite relative intensity differences from brain tissue samples of pigs exclusively fed bovine, caprine, or ovine milk for 15 days. Score plot of A) hippocampus B) prefrontal cortex and C) striatum.**

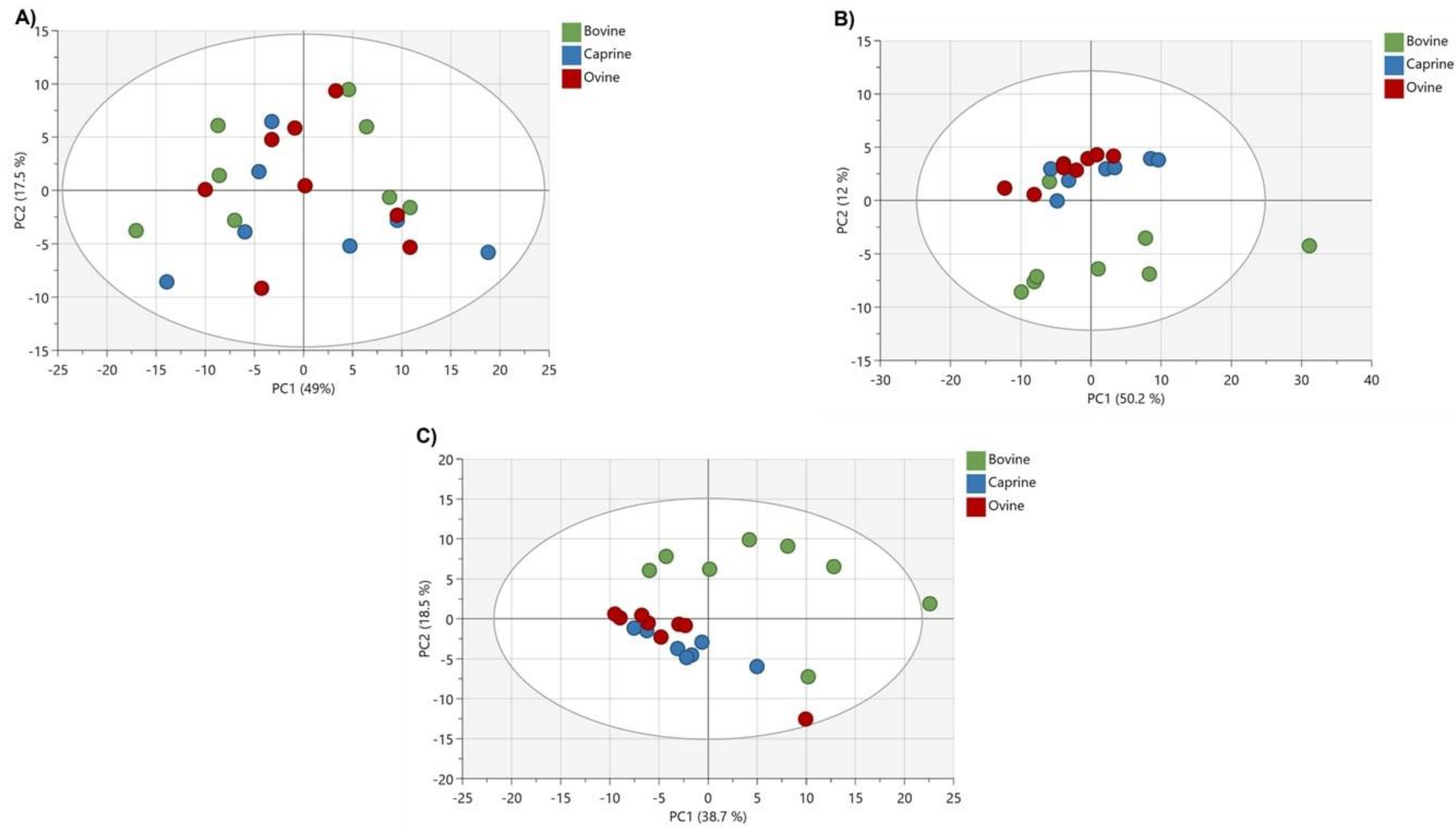



**Figure A4 Principal component analysis (PCA) showing lipid relative intensity differences from brain tissue samples of pigs exclusively fed bovine, caprine, or ovine milk for 15 days. Score plot of A) hippocampus B) prefrontal cortex and C) striatum**

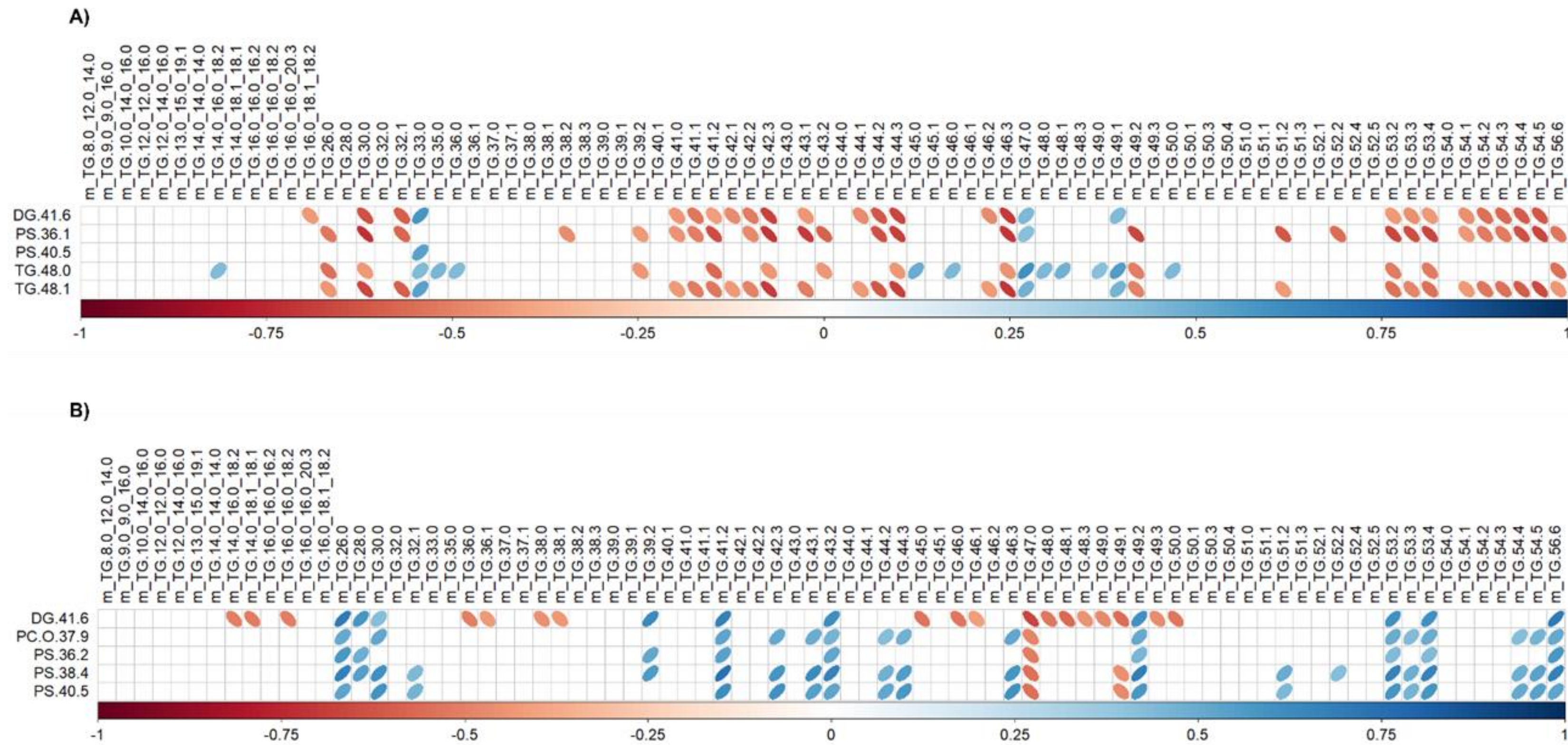
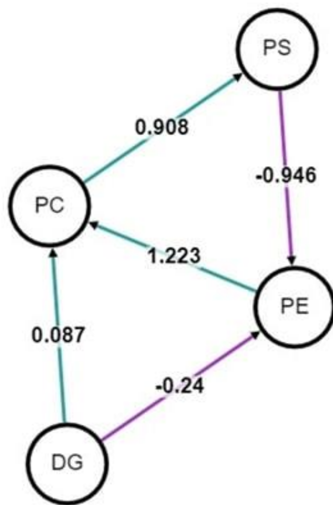



**Figure A5 Correlation plot showing Spearman correlations between milk intake (in %) and relative lipid intensities in (A) prefrontal cortex (n = 23), (B) hippocampus (n = 23) tissue samples obtained from piglets fed with milk from bovine, ovine or caprine species.**

The colour of the ellipse indicates the type of correlation, i.e., blue indicates a positive correlation, and red indicates a negative correlation. The ellipse shape indicates the magnitude of the correlation, i.e., the stronger the correlation, the flatter the ellipse. Only significant correlations ($P < 0.05$) are shown. The legend at the bottom of each correlation plot shows the correlation coefficients with their corresponding colours. The horizontal axis represents milk lipid intakes (preceded with the letter m), and the vertical axis represents brain lipid relative intensities.



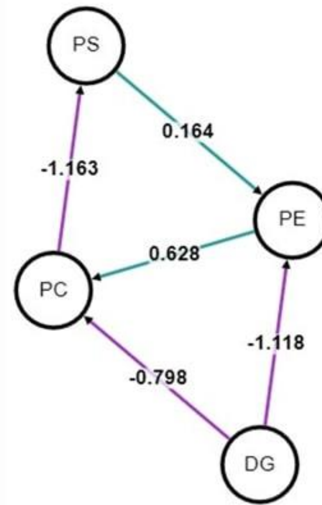




**Figure A6 Lipid metabolite network exported from BioPAN in the A) hippocampus of ovine vs caprine milk groups and B) striatum ovine vs caprine milk groups.**

Only the pathways with a P-value < 0.05 were considered significant (Z-score equivalent > 1.645).

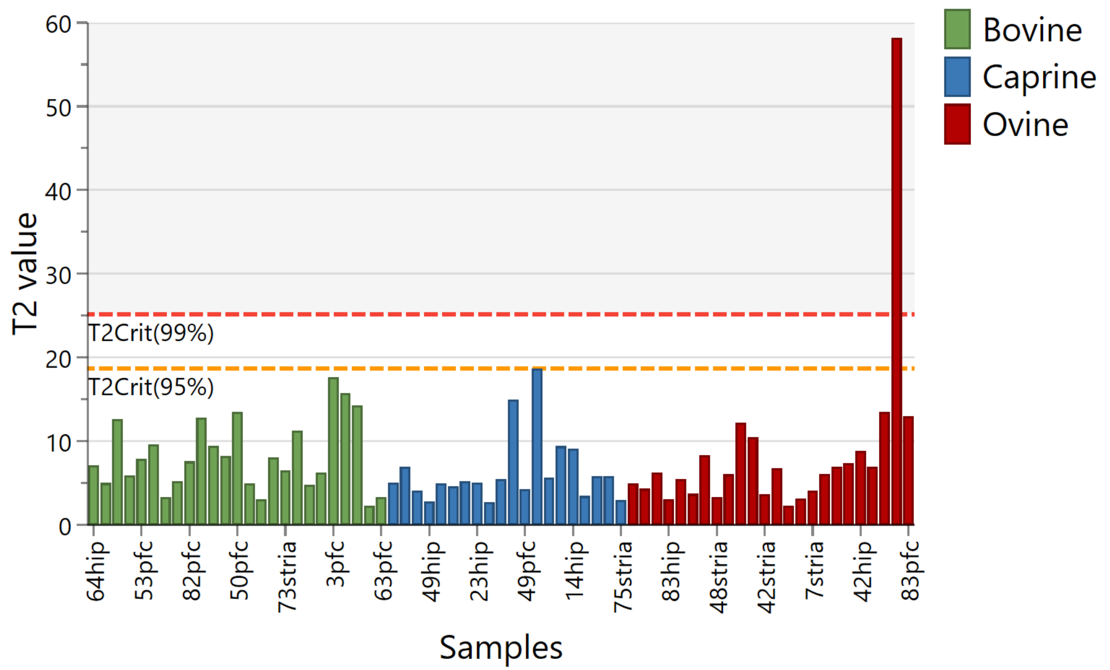



**Figure A7 Hotelling T2 plot showing potential outliers among the brain tissue samples of the piglets exclusively fed with bovine, caprine or ovine milk for 15 days.**

The red and orange dotted lines represent the 99% and 95% confidence intervals. A T2 value larger than the 99% confidence indicates that the sample is far away from other samples, and the probability of it belonging to the same class as other samples is lower than 1 %, hence an outlier.

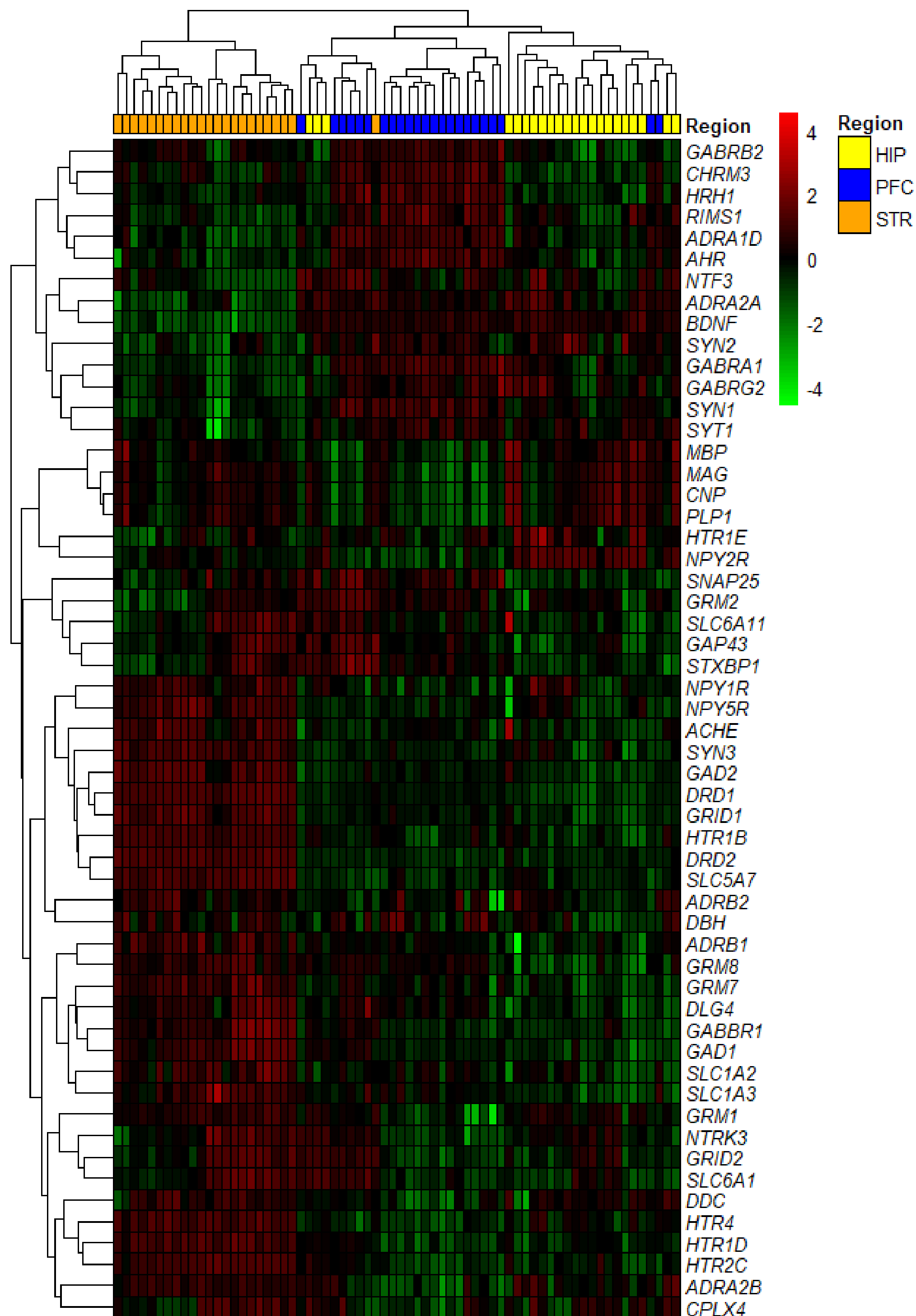



**Figure A8 Heatmap showing hierarchical clustering of significantly expressed genes between the hippocampus, prefrontal cortex, and striatum tissue samples of piglets fed milk from ovine, bovine, or caprine species.**

Heatmap colour indicates normalised (Z score) mRNA counts scaled across rows. The intensity of the red colour denotes the number of standard deviations above the mean (increased gene expression levels), and the intensity of the green colour denotes the number of standard deviations below the mean (decreased gene expression levels). HIP, hippocampus; PFC, prefrontal cortex; STR, striatum.

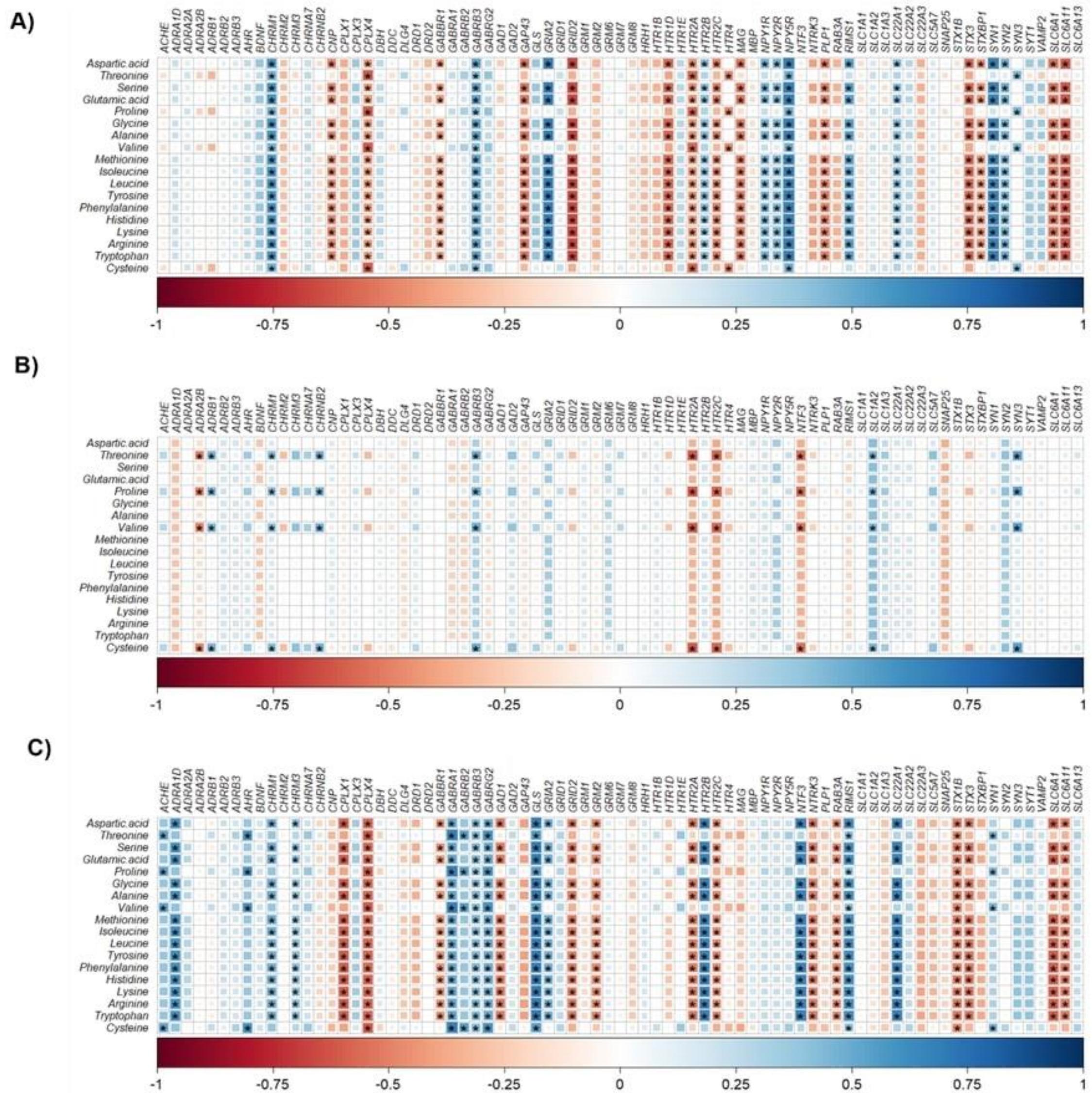



**Figure A9 Correlation plot depiction of the Spearman correlations between milk amino acid intake and gene expression levels in (A) hippocampus (n = 22), (B) prefrontal cortex (n = 23), and (C) striatum (n = 23) tissue samples obtained from piglets fed with milk from bovine, ovine or caprine species.**

The colour and size of the squares indicate the magnitude of the correlation, i.e., blue indicates a positive correlation, and red indicates a negative correlation. Asterisks indicate the significance of correlation ($P < 0.05$). The legend at the bottom of each correlation plot shows the correlation coefficients with their corresponding colours. n, number of samples used for correlation.

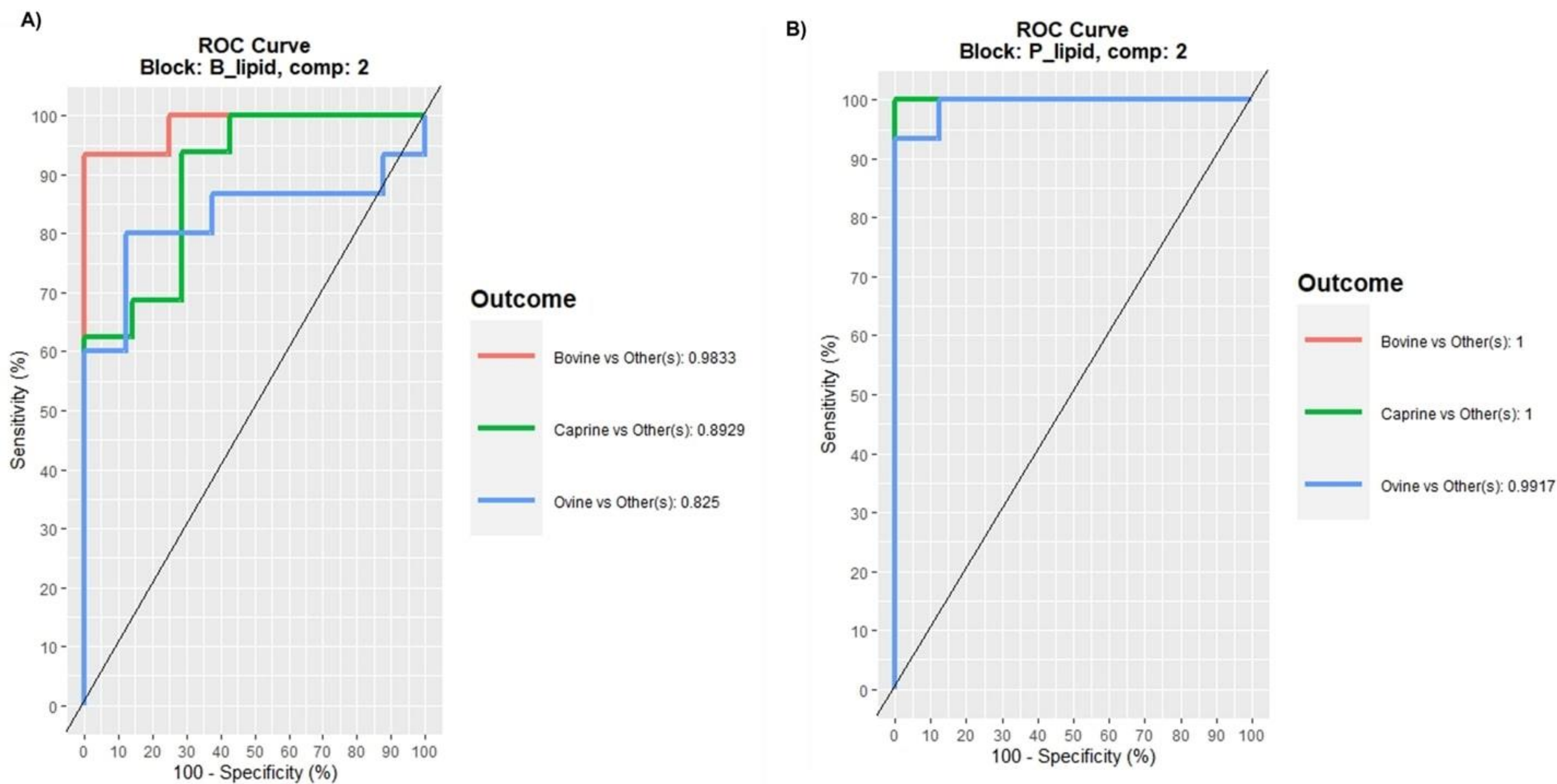
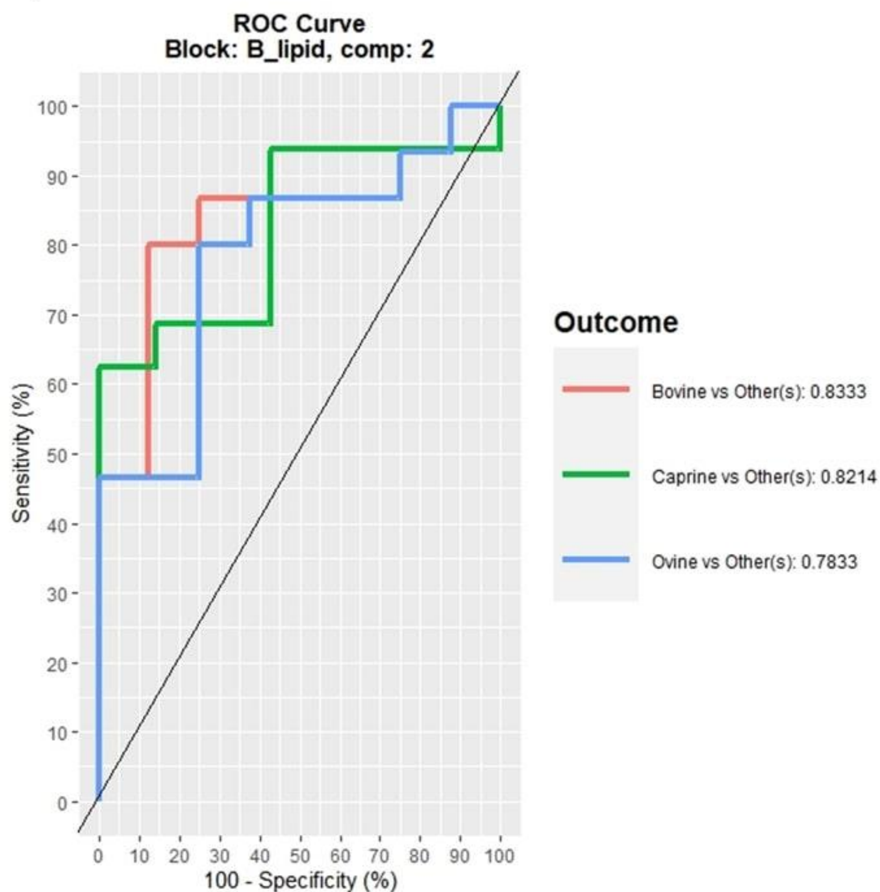



**Figure A10 ROC and AUC based on multiblock sPLS-DA performed on the A) hippocampal lipid relative intensities and B) plasma relative intensities data sets individually after 2 components. The outcome represents the ROC curve and AUC for one milk treatment class vs the others. B_lipid, brain_lipid; P_lipid, plasma lipid.**



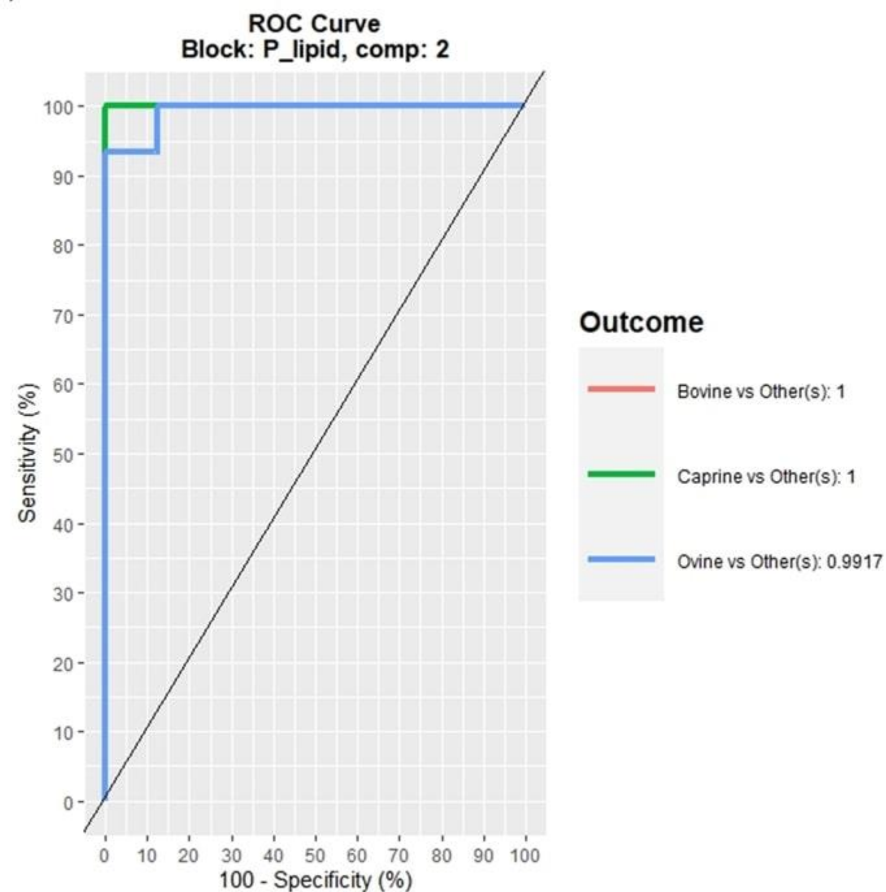
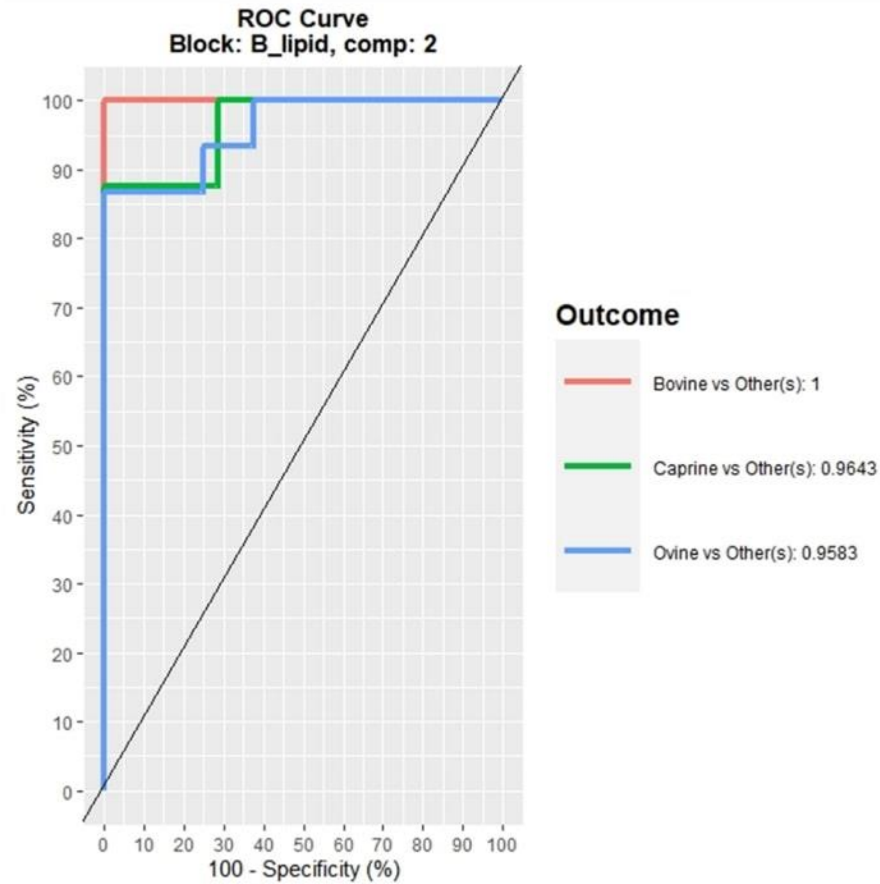




**Figure A11 ROC and AUC based on multiblock sPLS-DA performed on the A) prefrontal cortex lipid relative intensities and B) plasma relative intensities data sets individually after 2 components. The outcome represents the ROC curve and AUC for one milk treatment class vs the others. B_lipid, brain_lipid; P_lipid, plasma lipid.**

A)




B)

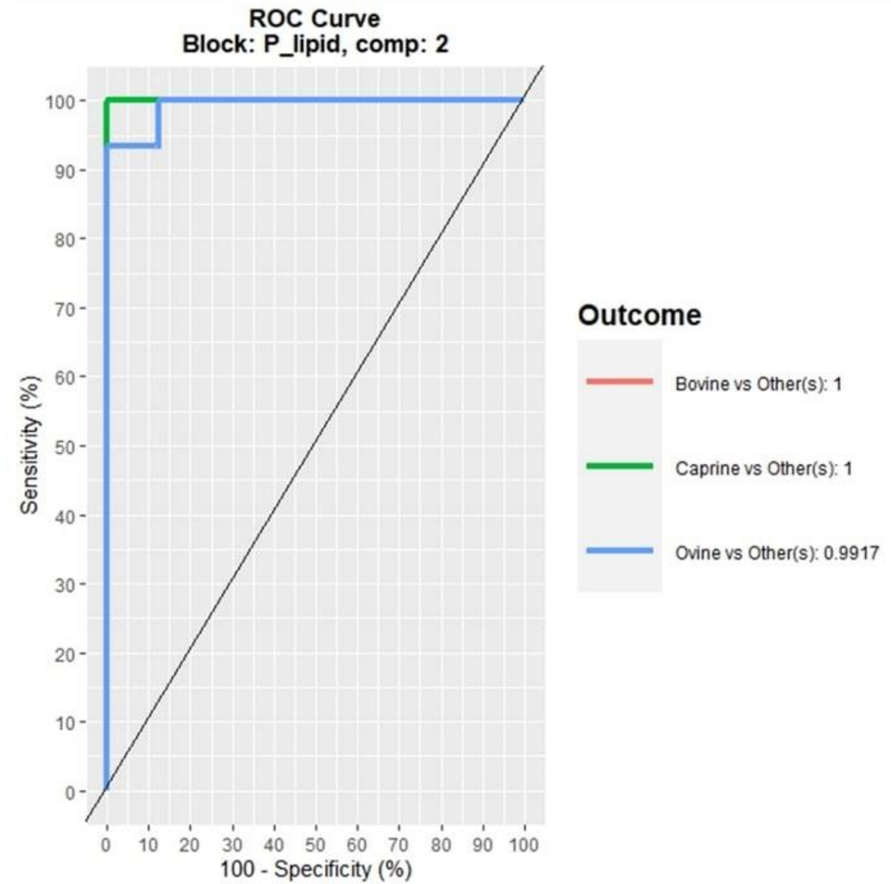



**Figure A12 ROC and AUC based on multiblock sPLS-DA performed on the A) striatal lipid relative intensities and B) plasma relative intensities data sets individually after 2 components. The outcome represents the ROC curve and AUC for one milk treatment class vs the others. B_lipid, brain_lipid; P_lipid, plasma lipid.**

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We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

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| Student name: | Ankita Jena |
| Name and title of main supervisor: | Prof Warren McNabb |
| In which chapter is the manuscript/published work? | Chapter 2 |
| What percentage of the manuscript/published work was contributed by the student? | 85 |

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The candidate performed the literature search, read and analysed the relevant literature, wrote the original draft, and reviewed and edited the manuscript.

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Jena A, Montoya CA, Mullaney JA, Dilger RN, Young W, McNabb WC and Roy NC (2020) Gut-Brain Axis in the Early Postnatal Years of Life: A Developmental Perspective. Frontiers in Integrative Neuroscience14:44. doi: 10.3389/fnint.2020.00044

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| Student name: | Ankita Jena |
| Name and title of main supervisor: | Prof Warren McNabb |
| In which chapter is the manuscript/published work? | Chapter 3 |
| What percentage of the manuscript/published work was contributed by the student? | 85 |

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

The candidate assisted with collecting biological samples, conducted laboratory experiments and data analysis, and wrote the original draft.
Plasma metabolites profiling of peripheral blood in response to ruminants' milk in early postnatal life
Jena A, Montoya CA, Fraser K, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC, & Roy NC
To be submitted: Frontiers in Nutrition

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| Name and title of main supervisor: | Prof Warren McNabb |
| In which chapter is the manuscript/published work? | Chapter 4 |
| What percentage of the manuscript/published work was contributed by the student? | 85 |

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

The candidate assisted with collecting biological samples, conducted laboratory experiments and data analysis, and wrote the original draft.
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Jena A, Fraser K, Montoya CA, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC, & Roy NC
To be submitted: Frontiers in Neuroscience

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

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|---|---|---|---|
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| Name and title of main supervisor: | Prof Warren McNabb |
| In which chapter is the manuscript/published work? | Chapter 5 |
| What percentage of the manuscript/published work was contributed by the student? | 85 |

Describe the contribution that the student has made to the manuscript/published work:
The candidate assisted with collecting biological samples, conducted laboratory experiments and data analysis, wrote the original draft, and reviewed and edited the manuscript.

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(●) **The manuscript/published work is published or in press**

Please provide the full reference of the research output:
Jena A, Montoya CA, Young W, Mullaney JA, Roy D, Dilger RN, Giezenaar C, McNabb WC and Roy NC (2022) The effects of ruminant milk treatments on hippocampal, striatal, and prefrontal cortex gene expression in pigs as a model for the human infant. Frontiers in Neuroscience 16:937845. doi: 10.3389/fnins.2022.937845

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