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RAYLLANA LARSEN

OMICS APPROACHES IN NUTRITIONAL MODULATION IN SWINE: ANALYSES OF LIPIDOMICS AND MICROBIOME IN WEANED PIGLETS AND NUTRIGENOMICS IN LACTATING SOWS

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Tese apresentada ao Programa de Pós-graduação em Ciência Animal, da Universidade do Estado de Santa Catarina, como requisito parcial à obtenção do título de Doutora em Ciência Animal, área de concentração em Produção Animal.
Orientador: Prof. Dr. Dimas E. de Oliveira.
Co-orientador: Dr. Daniel E. Rico.

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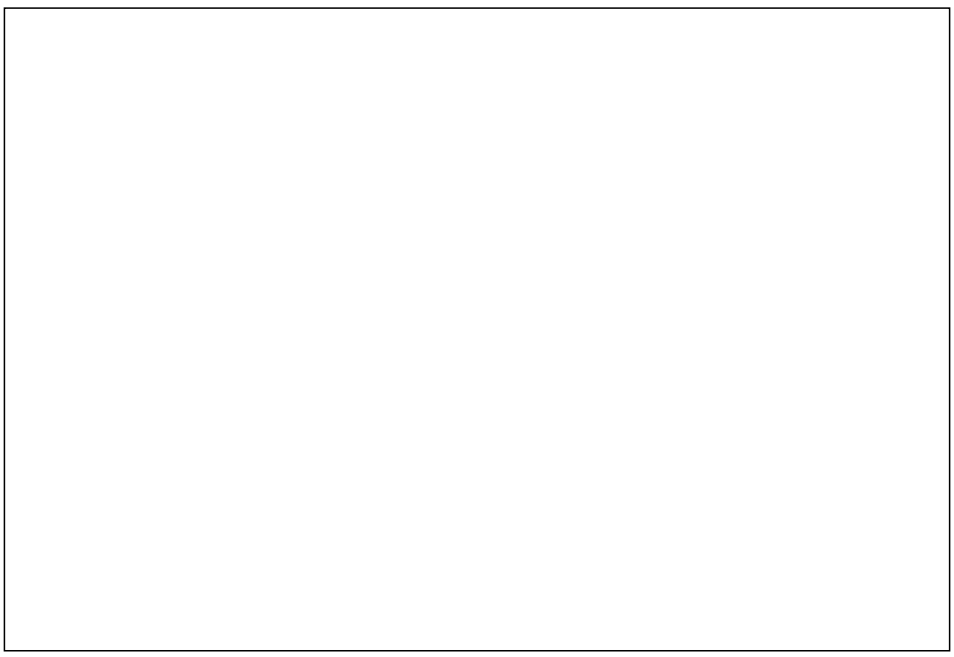

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Dedico esse trabalho aos que, silenciosamente,
plantam sonhos em mentes inquietas.

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“The task is to live your life in such a way that you must wish to live it again - for you will anyway […] Only make sure you come to know what gives you the highest feeling and then spare no means. Eternity is at stake!”

(Friedrich Nietzsche)

RESUMO

LARSEN, Rayllana. Abordagens ômicas na modulação nutricional em suínos: análises de lipidômica e microbioma em leitões desmamados e nutrigenômica em matrizes suínas lactantes. p.131. Tese (Doutorado em Zootecnia – Área: Produção Animal) – Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência Animal, Lages, 2025.

Lipídios polares e tiazolidinediona (TZD) podem ser utilizados como ferramentas para a modulação nutricional na produção suína. Os lipídios polares, derivados de subprodutos lácteos, têm propriedades anti-inflamatórias e influenciam perfis lipidômicos plasmáticos e a microbiota intestinal em leitões. Enquanto a TZD, como agonista do peroxisome proliferator-activated receptors gamma (*PPARG*), pode regular a expressão gênica relacionada à síntese de lipídios e proteínas em porcas lactantes. Esta tese emprega abordagens ômicas—nutrigenômica, lipidômica e microbiômica—para explorar seu potencial na nutrição suína. No segundo capítulo, os efeitos da suplementação de TZD foram avaliados em matrizes lactantes. Quarenta porcas multíparas foram distribuídas aleatoriamente em dois tratamentos: (1) Controle (sem TZD) ou (2) 8 mg/kg de peso corporal de TZD na dieta, administrados do 13º ao 28º dia de lactação. A suplementação com TZD aumentou a expressão de *AGPAT6* (+20%) e *CSN3* (+43%) no tecido mamário, genes essenciais para a síntese de lipídios e proteínas. Além disso, reduziu ácidos graxos saturados (SFA) e aumentou ácidos graxos monoinsaturados (MUFA) no leite, melhorando seu perfil nutricional sem alterar insulina plasmática, glicose ou ácidos graxos não esterificados. Os terceiro e quarto capítulos abordam os efeitos dos lipídios polares em leitões desmamados, integrando lipidômica e microbiômica para avaliar seu impacto. Ambos derivam de um único experimento com 240 leitões (21 dias de idade; 6,3 ± 0,5 kg), distribuídos aleatoriamente em dois grupos dietéticos: uma dieta vegetal rica em lipídios neutros da soja (SD) ou uma dieta rica em lipídios polares derivados de subprodutos de queijo (PD). Dentro de cada dieta, os animais receberam um dos três sucedâneos lácteos (MR) durante os primeiros sete dias pós-desmame: (1) MR comercial com lipídios de origem animal e coco (CO), (2) MR à base de lipídios polares (PO) ou (3) MR à base de lipídios de soja (SO). No terceiro capítulo, análises lipidômicas revelaram que a dieta PD aumentou os níveis plasmáticos de esfingolipídios (+15%), fosfolipídios (+10%) e ésteres de colesterol (+8%) em comparação à dieta SD, que elevou os glicerolipídios contendo ácidos graxos de 18 carbonos (+12%). Além disso, PO e CO aumentaram os níveis de ceramidas (+20%) e ésteres de colesterol (+15%), demonstrando o efeito modulador dos lipídios polares no metabolismo lipídico dos leitões. No quarto capítulo, a dieta DP alterou significativamente a composição da microbiota, aumentando gêneros específicos associados a *Firmicutes* (*Coprococcus, Roseburia*) e modulando mediadores lipídicos plasmáticos. Leitões alimentados com PD apresentaram níveis mais altos de endocanabinoides (AEA, 2-AG), enquanto a dieta SD aumentou mediadores lipídicos pró-inflamatórios (13-HODE, 13-KODE) derivados do ácido linoleico. Esses resultados destacam as propriedades anti-inflamatórias dos lipídios polares e seu potencial para melhorar a saúde intestinal e reduzir o estresse do desmame. Essas descobertas ressaltam o potencial da integração entre nutrigenômica, lipidômica e microbiômica para otimizar a função metabólica, a saúde intestinal e o desempenho produtivo, oferecendo estratégias inovadoras para uma produção suína mais sustentável e eficiente.

Palavras-chave: lipídios polares; tiazolidinediona; lipidômica plasmática; diversidade da microbiota intestinal.

ABSTRACT

LARSEN, Rayllana. Omics approaches in nutritional modulation in swine: analyses of lipidomics and microbiome in weaned piglets and nutrigenomics in lactating sows. p.131. Thesis (Doctorate in Animal Science – Area: Animal Production) – Santa Catarina State University. Post-Graduation Program in Animal Science, Lages, 2025.

Polar lipids and thiazolidinedione (TZD) have emerged as promising tools for nutritional modulation in swine production. Polar lipids, derived from dairy by-products, influence plasma lipidomic profiles and gut microbiota, while TZD, as a peroxisome proliferator-activated receptors gamma (*PPARG*) agonist, regulates gene expression related to lipid and protein synthesis. This thesis employs omics approaches—nutrigenomics, lipidomics, and microbiomics—to explore their potential in swine nutrition. In the second chapter, the effects of TZD supplementation were evaluated in lactating sows. Forty multiparous sows were randomly assigned to two treatments: (1) Control (no TZD) or (2) 8 mg/kg BW of TZD mixed with feed, administered from day 13 to 28 of lactation. TZD increased the expression of *AGPAT6* (+20%) and *CSN3* (+43%) in mammary tissue, which are key genes involved in lipid and protein synthesis. Furthermore, TZD reduced saturated fatty acids (SFA) and increased monounsaturated fatty acids (MUFA) in milk, improving its nutritional profile without altering plasma insulin, glucose, or non-esterified fatty acids. These findings highlight TZD's potential to enhance metabolic activity and milk composition through nutrigenomic modulation. The third and fourth chapters focus on the effects of polar lipids in weaned piglets, integrating lipidomics and microbiomics to evaluate their impact. Both chapters derive from a single experiment involving 240 piglets (21 days old; 6.3 ± 0.5 kg) randomly assigned to two diet groups: a plant-based diet rich in neutral lipids from soybean (SD) or a polar lipid-rich diet from cheese by-products (PD). Within each diet, animals received one of three milk replacers (MR) during the first seven days post-weaning: (1) commercial MR with animal and coconut lipids (CO), (2) polar lipid-based MR (PO), or (3) soybean lipid-based MR (SO). In the third chapter, lipidomics analyses revealed that the PD diet increased plasma sphingolipids (+15%), phospholipids (+10%), and cholesterol esters (+8%) compared to SD, which elevated glycerolipids containing 18-carbon fatty acids (+12%). Additionally, PO and CO increased ceramides (+20%) and cholesterol esters (+15%), demonstrating the modulatory effects of polar lipids on piglet lipid metabolism. In the fourth chapter, the PD diet significantly altered microbiota composition, increasing specific *Firmicutes*-associated genera (*Coprococcus, Roseburia*) while also modulating plasma lipid mediators. PD-fed piglets had higher levels of endocannabinoids (AEA, 2-AG), whereas the SD diet increased pro-inflammatory lipid mediators (13-HODE, 13-KODE) derived from linoleic acid. These results highlight the anti-inflammatory properties of polar lipids and their potential to improve intestinal health and alleviate weaning stress. These findings highlight the potential of integrating nutrigenomics, lipidomics and microbiomics to optimize metabolic function, gut health and performance, offering innovative strategies for sustainable swine production.

Keywords: polar lipids; thiazolidinedione; plasma lipidomics; gut microbiota diversity.

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ABBREVIATIONS LIST

2-AG - 2-arachidonylglycerol

AA - arachidonic acid

ACACA – Acetyl-CoA Carboxylase Alpha

AEA - *N*-arachidonoylethanolamine

AGPAT – 1-Acylglycerol-3-Phosphate O-Acyltransferase

AGPAT6 – 1-acylglycerol-3-phosphate O-acyltransferase 6

AKT – Protein Kinase B

ALA - alpha-linolenic acid

ATGL – Adipose Triglyceride Lipase

B4GALT – Beta-1,4-Galactosyltransferase

BCAAs – Branched-Chain Amino Acids

BCS – Body Condition Score

BW – Body Weight

CB_1 - Cannabinoid receptor 1

CB_2 - Cannabinoid receptor 2

CD36 – Cluster of Differentiation 36

CE – Cholesteryl ester

Cer – Ceramide

CO – Coconut lipids milk replacer

COXs - cyclooxygenases

CSN1S1 – Casein Alpha S1

CSN2 – Casein Beta

CSN3 – Casein Kappa

CYPs - cytochrome P450

DAG – Diacylglycerol

DG – Diacylglyceride

DGAT – Diacylglycerol O-Acyltransferase

DGAT1 – Diacylglycerol O-acyltransferase 1

DGLA - dihomo-g-linolenic acid

DNA - Deoxyribonucleic acid

DPA n-6 - docosapentaenoic acid from the n-6 pathway

eCBome - endocannabinoidome

ELISA – Enzyme-linked immunosorbent assay

ETA - eicosatetraenoic acid

FA – Fatty acids

FABP – Fatty Acid-Binding Protein

FASN – Fatty Acid Synthase

FDR - false discovery rate

GLUT1 – Glucose Transporter 1

GLUT4 – Glucose Transporter 4

GPAT – Glycerol-3-Phosphate Acyltransferase

Hex2Cer – Ceramide with two hexose sugar residues attached to the sphingoid base and a fatty acid chain

Hex3Cer – Ceramide with three hexose sugar residues attached to the sphingoid base and a fatty acid chain

HexCer – Ceramide with a hexose sugar residue attached to the sphingoid base and a fatty acid chain

HPLC – High-performance liquid chromatography

HSL – Hormone-Sensitive Lipase

IL-1β – Interleukin-1 Beta

IL-6 – Interleukin-6

LA - linoleic acid

LALBA – Alpha-Lactalbumin

LBP - Lipopolysaccharide binding protein

LC-MS/MS - Liquid chromatography-mass spectrometry/mass spectrometry

LDA - linear discriminant analysis

LEfSe - linear discriminant analysis effect size

LOXs – lipoxygenases

LSMEANS – Least-squares means

Lyso – Lysophosphatidylcholine

ME – Metabolizable Energy

MR – Milk replacer

mTOR – Mechanistic Target of Rapamycin

NEFA – Non-Esterified Fatty Acids

NF-κB – Nuclear Factor Kappa B

PC – Phosphatidylcholine

PCaa – Phosphatidylcholine with an acyl chain

PCae – Phosphatidylcholine with an acyl-alkyl chain

PCoA - Principal coordinates analysis

PCR - Polymerase chain reaction

PD – Polar diet

PEA – palmitic acid

PLS-DA - partial least squares discriminant analysis

PO – Polar lipids milk replacer

PPARG – Peroxisome Proliferator-Activated Receptor Gamma

PUFA – Polyunsaturated fatty acids

RNA - Ribonucleic acid

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

RPS18 – Ribosomal protein S18

S1P – Sphingosine-1-phosphate

SCD – Stearoyl-CoA Desaturase

SD – Soy diet

SEA – stearic acid

SL - sphingolipids

SM – Sphingomyelin

SM(OH) – Sphingomyelin with a hydroxyl group

SO – Soy lipids milk replacer

SREBP1 – Sterol Regulatory Element Binding Protein 1

STAT5 – Signal Transducer and Activator of Transcription 5

TG – Triglyceride

TNF-α – Tumor Necrosis Factor Alpha

TZD – Thiazolidinedione

VIP - variable importance projection

SUMMARY

INTRODUCTION

According to USDA projections, global pork consumption is expected to grow by 17.3% between 2021 and 2030, with Brazil, the 4th largest producer and exporter, anticipating a 25.8% increase in production during the same period (USDA, 2021; MAPA, 2021). To meet this demand, Brazilian pig farming leverages advancements in genetics, nutrition, health, and management.

Since the 1970s, genetic improvement programs have focused on improving productive traits and creating commercial lines with a higher number of live-born and weaned piglets, higher litter weights and better carcass traits, greater robustness and resistance to certain pathogens (FÁVERO & FIGUEIREDO, 2009; FIGUEIREDO et al., 2016). The use of animal genotyping has accelerated genetic gains within breeding programs by up to 50% (WEI et al., 2022). However, these strategies have also triggered other challenges in pig farming that are currently the basis of research, such as insufficient milk production from sows for larger litters.

Genetics is not the only way to improve animal productivity and performance; nutrition plays an important role in this regard. In addition to feeding representing 73% of the farm's total production costs, it is among the main factors for the animal to express all its productive potential (EMBRAPA, 2025). The swine diet is based on a mix of cereals, ingredients of animal origin, minerals, vitamins, amino acids, and additives and must be formulated according to the requirements of each phase of the animal (NRC, 2012). Dietary composition can improve weight gain, change carcass quality, influence the immune system, and promote animal welfare. Thus, dietary strategies from the farrowing phase are developed to meet the different demand of animals and allow their maximum performance from a young age, which indirectly improves feed efficiency, serves the market, and reduces production costs.

In addition to genetics and nutrition, the environment, management, and health status are fundamental for complete animal performance and productivity. For this, biosecurity measures and highly technical production systems with a controlled environment are used (EMBRAPA, 2003). The most widely adopted type of management in the country is the intensive system of confined pig farming (ABCS, 2011). This system is usually divided into 3 segments: 1) Piglet Production Unit, responsible for reproduction, maternity and weaning of piglets; 2) Nursery Unit, growth phase of piglets after weaning; and 3) Finishing Unit, responsible for fattening the

animal and comprising the departure of pigs from the nursery until the slaughter phase. Segments and methods can be adapted according to farm availability or industry organization; however, the main objective of this system is to reach the final product with high quality in the shortest possible time (PEDERSEN, 2018). Because of this, strategies such as reducing the weaning age to reduce the gestation interval and increase the number of piglets produced per sow per year are widely adopted. On the other hand, these managements trigger new challenges related to welfare issues that reduce animal productivity (MAES et al., 2022). The stress generated in the animals by early weaning and adaptation to the nursery environment made this phase the most critical of this rearing system.

Even if each segment has its own speciality, they are interdependent for the effective success of the production system. This means that the performance of the litter since the farrowing ward directly influences in the productivity of the adult pig (QUINIOU et al., 2002). In this sense, mitigating the challenges that reduce the piglets' productive capacity through genetics, diet and/or management is of interest to the entire production chain.

To assist in research aimed at developing swine production, omics analyses can be applied to clarify biological processes, increase production and reproduction rates, improve the quality of animal products, promote animal growth and improve the efficiency of nutrient intake. In this context, this study used omics analyses to assist in nutritional modulations of swine in: 1) Piglet Production Unit, through nutrigenomic analysis of a synthetic molecule added to the diet, aiming to improve the composition of sow milk in the farrowing ward; and 2) Nursery Unit, evaluating changes in the lipidomic profile and composition of the intestinal microbiome of weaned piglets subjected to nutritional strategies to reduce weaning stress.

CHAPTER I
LITERATURE REVIEW

1 FACTORS THAT AFFECT THE GROWTH OF PIGLETS

As mentioned in the introduction, the bottleneck in pig production is found in the factors that affect piglet performance. Although the transition phase of animals from the Pig Production Unit to the Nursery Unit is the most critical, within these segments there are also factors that can impact the expression of the animals' genetic potential. To better discuss these effects, we classified the challenges into before and after weaning.

1.1 PREWEANING CHALLENGES

Piglet care begins during pregnancy. The health, management, well-being and nutrition of the sow directly interfere with the number of piglets produced/female/year, survival rate at birth and the health of the newborns. The mortality rate of piglets at farrowing can reach 10% (ABRAHÃO et al. 2004), while from postpartum to weaning, the Brazilian average is 5 to 15% (FARIA, 2019).

The main causes of mortality in the maternity ward are crushing, hypothermia, diarrhea, localized infections that can progress to septicemia, hypoglycemia, piglets born with low birth weight and low consumption of mammary gland milk. Most of these factors can be resolved through management, with care in the whelping box, management of heat sources, hygiene, and vaccinations. However, the ingestion of colostrum and milk to obtain nutrients and passive immunoglobulins from the sow is what guarantees the resistance to pathogens and the good performance of the newborns until weaning. In this sense, in addition to postpartum management, the sow's potential to meet litter requirements can be a limiting factor at this initial stage (KING, 2000).

1.1.1 Limitations in milk production and composition

Advances in genetics, combined with management innovations, have resulted in significant increases in litter size in commercial pig production (BRUNS et al., 2018). From 2006 to 2019, the total number of pigs born per litter increased by 4.5 (TOKACH et al, 2019). In contrast, although the suckling movement of the piglet plays a role in increasing mammary gland mass (PARK, 2011), the milk production and composition of highly prolific sows has been shown to be insufficient to achieve maximum litter growth (NOBLE et al. al.al., 2002).

Modern sows can produce 10 to 12 kg milk per day and the peak production occurs at 21 days of lactation (HAERNE, 2007). Piglets suckled by high-performance sows grow on average approximately 250 g/d (HOJGAARD et al., 2019), however, the biological potential of artificially reared young animals with *ad libitum* access to milk replacers can reach more than 450 g/d (HARRELL et al., 1993).

A study by Zhang et al. (2018) revealed that in the last 30 years, only small changes in sow milk were reported, with an increase of 1% in fat content (7.5%), reduction of 1% in lactose level (5%) and no change in protein (5%). Another study revealed that the size of the litter does not regulate the total milk production of the sow (DEVILLERS et al., 2007). That is, if the sow produces the same amount and composition of milk, the larger the litter, the more restricted the individual intake of milk nutrients and consequently, less heavy the piglets wean. If the animal reaches the weaning age lighter, it may be more vulnerable to the challenges that this period entails.

1.2 WEANING AND NURSERY CHALLENGES

In nature, the weaning is a gradual process that takes place over 14 to 17 weeks (JENSEN, 1986) and coincides with a complete maturation of gastrointestinal (GI) epithelial, immune, and nervous systems that ease the stress of feeding and behavioral changes for the piglet. In contrast, in an intensive production system, the weaning is abrupt and occurs between 14 and 30 days of age, in a period of diminished passive immunity from sow's milk and a not fully developed GI in piglet (MOESER, et al. 2017).

The weaning period requires the piglet to adapt from sow milk to a less digestible and palatable dry solid diet, leading to a reduction in feed intake. Already on the first day after weaning, pigs lose about 100-250 g body weight (BW) and metabolizable energy (ME) intake in the first week post-weaning can be reduced by 30 to 40% compared to preweaning ME intake (LE DIVIDICH & SEVE, 2000). The extent and duration of underfeeding are variable, however, the severity of weight loss after weaning and the recovery time impact animal performance and total days to market (KATS et al., 1992).

The change in the diet type and the amount of feed intake during this period induces increases in relative weight of up to 52% in the small intestine and 30% in the pancreas in 14 days post weaning (LE DIVIDICH & SEVE, 2000). This enlargement in gastrointestinal tract is associated to changes in the small intestine structure that

compromises the function of the intestinal barrier. Alterations such as villous atrophy and crypt elongation (PLUSKE et al., 1997); reductions in digestive enzymes and transient decreases in pancreatic secretions (LALLES, 2010; LACKEYRAM et al., 2010) may affect the digestive, absorptive, and secretory capacity of the small intestine. Since the epithelium is the first physical barrier of protection in the intestine, its structural degeneration and atrophy increases permeability and allows bacteria and toxins to cross this barrier. As result, there is malabsorption, diarrhea, and inflammation which further compromises animal growth and performance (CAMPBELL et al., 2013).

Immunological and hormone responses to weaning are also detrimental to the animal's metabolism. Activation of stress pathway though increases in serum cortisol and catecholamine concentrations in weaning pigs have negative effect in the immune system, supressing lymphocyte proliferation to mitogens, natural killer cell activity and neutrophilic chemotaxis leading to intestinal inflammation via brain-gut axis activation (PLUSKE et al. 2019). Furthermore, an up-regulation of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α affects intestinal integrity and epithelial function, contributing to metabolism disorders and post-weaning diarrhea (PIÉ et al. 2004).

Changes in energy metabolism or in immune response create an imbalance between generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and their removal by antioxidants and result in the oxidation of biological molecules. Oxidative stress activates the NF-κB nuclear transcription, the main regulator of inflammation, which stimulates the production of oxidants and enhances oxidative stress (PANTANO et al., 2006). In weaned piglets, the oxidative stress negatively affects the integrity of intestinal barrier and the resistance to diseases (BUCHET et al. 2017).

The composition of the microbiota is also affected by stress. Li et. al (2018) observed that 19 species of microbes increased and 4 decreased in the intestine of piglets after weaning. Furthermore, the metabolic pathways of phenylalanine metabolism, citrate cycle, glycolysis or gluconeogenesis, propanoate metabolism, nicotinate and nicotinamide metabolism have been shown to be involved in gut microbiota dysbiosis induced by weaning stress. Jiang et al. (2020) also investigated the effects of weaning stress on the colonic microbiome in piglets and the relationships between microbiome and metabolites; they found associations such as the colonic phylum *Bacteroidetes* with the carbohydrate metabolic pathway, the genus

Lactobacillus with the amino acid metabolic pathway, and the *Prevotellaceae-NK3B31* related to amino acid, carbohydrate, lipid and nucleotide pathways. More studies are needed to clarify these mechanisms and their correlations.

In summary, early weaned pigs are able to survive and overcome the stress of weaning. However, the separation from the sow combined with new environment, social status and dietary transition are psychosocial and immunological stressors that can reduce their state of health (ZHENG et al. 2021). Reduces in feed intake (LE DIVIDICH & SEVE, 2000), increases in intestinal permeability (WIJTTEN et al. 2011), oxidative stress (ZHU et al. 2012), and activation of immune system (KICK et al. 2012) affect the growth performance during this period (MCCRACKEN et al., 1999).

In both cases, farrowing or nursery phases, diet is a key factor in meeting these challenges. In the Pig Production Unit, looking for alternatives that improve composition of milk and its quantity through the genetic selection and diet of the sows can be an effective strategy in production systems that have as a performance limit the insufficient production of milk by the sow for the number of piglets born at each farrowing, or that aim to increase the weight of piglets at weaning. While in the weaning and Nursery Unit adaptation period, dietary strategies can reduce the impacts of biological stress on animals, ensuring greater performance and productivity of piglets.

2 NUTRITIONAL STRATEGIES TO IMPROVE COLOSTRUM AND MILK QUALITY

Colostrum production in sows is concentrated between two to three days before parturition and two days after; and is then gradually replaced by milk production. This phase comprises the transition period where there is also partition of nutrients for fetal growth, mammary tissue, and uterine components (FEYERA and THEIL, 2017). Concomitantly, sows transition from a gestation diet - with a higher concentration of fiber - to a lactation diet, with greater energy density and rich in lysine, which stimulates protein synthesis and lactation. Despite the period of adaptation to the new diet that the metabolism undergoes, studies have shown an increase in the protein and fat content of colostrum in sows supplemented with lipids, proteins (WANG et al., 2016; WIECEK et al., 2018) and amino acids (TOKACH et al., 2019) in the last third of pregnancy.

Lactation represents about 15% to 20% of the sow's productive cycle (approximately 21 days out of a total of 142), and it is the most metabolically

demanding production phase (TOKACH et al., 2019). Milk production represents up to 80% of the energy required by a lactating sow and, when not met by voluntary intake, body fat and protein reserves are mobilized leading to a negative energy balance (NATIONAL RESEARCH COUNCIL, 2012). Dietary strategies have been developed to improve feed intake and reduce catabolism at this stage.

Energy intake can be modified using fats, oils and fibers. The average fat content in the diet of lactating sows varies between 3% and 6% in dry matter, which allows an increase in dry matter intake with consequent improvement in the sow's energy balance and can result in weight gain in the litter through increased milk fat concentration (ROSERO et al., 2015); On the other hand, highly fibrous diets have low energy value and restrict intake, being less used in lactation diets (SCHOENHERR et al., 1989).

Lactating sows utilize up to 70% of dietary protein for milk protein synthesis (PEDERSEN et al., 2016). Therefore, providing a diet balanced in essential amino acids and with a minimum digestible crude protein (CP) content of 13.5% to 14.3% improves milk quality and reduces sow body weight losses during lactation (STRATHE et al., 2017; PEDERSEN et al., 2019). Amino acid requirements are variable according to the mobilization of body tissues during lactation. Lysine has been shown to be the first limiting amino acid and its requirement is predicted to be around 13 g/d of mobilization of body protein reserves and 27 g/d for each 1 kg of litter growth (TOKACH et al., 2019). In addition, the branched-chain amino acids (BCAAs) isoleucine, leucine and valine provides an effective means to improve milk synthesis by lactating sows and, in turn, the growth and survival of piglets (REZAEI et al., 2022).

Studies at the molecular level have clarified the role of dietary supplements on hormones that regulate milk production as well as the coding of genes responsible for the synthesis of milk components in production animals (BIONAZ, 2012; MENEZIES et al., 2009).

2.1 THE ROLE OF INSULIN IN MILK SYNTHESIS

Among the hormones that regulate the synthesis of milk components, insulin has been shown to be fundamental for the synthesis of proteins and, by promoting the intracellular flow of glucose, it has the capacity to alter the levels of lactose in milk (GRIINARI et al, 1997; BIONAZ, 2012).

Insulin influences, by direct and indirect mechanisms, the two main steps in protein regulation: the control of protein gene expression via activation of the transducer and activator of transcription signal 5A (STAT5) through increased phosphorylation of transcription factor E74 (MENEZIES et al., 2009; BIONAZ and LOOR, 2011) and; the control of the regulation of the amount of translation through the activation of the mammalian target protein of rapamycin (mTOR) (PROUD, 2007; BURGOS et al., 2010). *In vitro* studies with rodent (CHOI et al., 2004) and bovine (MENEZIES et al., 2009) mammary explants have demonstrated that insulin increases casein synthesis and is essential both for mRNA translation and for transcription of the β-casein. In addition, insulin has a stimulating action on sterol regulatory element binding proteins (SREBPs), a family of transcription factors that regulate lipid homeostasis by directly activating the expression of genes involved in the synthesis and uptake of fatty acids, triglycerides, phospholipids and cholesterol (HORTON, 2002).

Although the mechanisms involved in these actions are not fully understood, it is known that peroxisome proliferator-activated receptors gamma (PPARG) can positively regulate insulin sensitivity (BROWN et al., 2003; EVANS et al., 2004) and play a key role in adipogenesis of adipose tissue (HOSSEINI et al., 2015). Since then, PPARG agonists have been used to reverse insulin resistance in humans with type 2 diabetes or impaired glucose tolerance and to combat obesity (REYNOLDS and GOLDBERG, 2006).

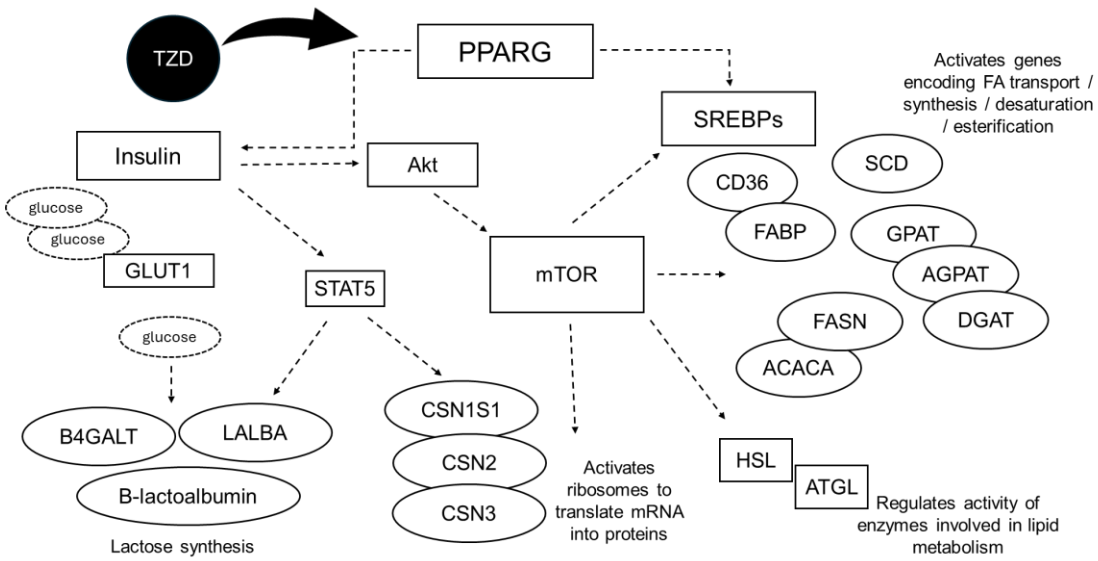
2.1.1 Thiazolidinedione

Thiazolidinedione (TZD) is a PPARG synthetic agonist that can stimulate cell insulin signaling by increasing protein synthesis via the mammalian target protein of rapamycin (*mTOR*) as well as acting on the family of transcription factors responsible for lipid homeostasis – the sterol regulatory element binding protein (*SREBP1*), altering the expression of genes related to the transport, synthesis, desaturation and esterification of fatty acids in adipose and mammary gland tissue (BIONAZ et al., 2013).

Pathways and genes related to lactose and protein synthesis and lipid modulation that TZD can alter in mammary gland tissue are represented in Figure 1. In summary, TZD enhances insulin activity, increasing glucose uptake in insulin-sensitive tissues (i.e. adipose tissue) through GLUT4. In mammary gland tissue during lactation, glucose uptake is mediated primarily by GLUT1, a basal glucose transporter,

independent of insulin action. GLUT1 ensures a continuous supply of glucose to the mammary gland, which is critical for lactose synthesis. Insulin activates the *mTOR* pathway through Akt, promoting ribosome activity, which translates mRNA into proteins and regulates key enzymes involved in lipid metabolism, such as HSL (hormone-sensitive lipase) and ATGL (adipose triglyceride lipase). Additionally, insulin acts on STAT5, a transcription factor that induces the synthesis of caseins (*CSN1S1, CSN2, CSN3*) and activates *LALBA* (alpha-lactalbumin). The possible increase in glucose transported by GLUT1, along with the activity of *LALBA, B4GALT1* (beta-1,4-galactosyltransferase), and beta-lactoglobulin, drives lactose synthesis in the mammary gland. Furthermore, *PPARG* can play a pivotal role in lipid metabolism by regulating the family of transcription factors involved in lipid homeostasis, particularly the *SREBP1*. Through *SREBP1, PPARG* can influence the expression of genes involved in lipid transport: *CD36* (cluster of differentiation 36) and *FABP* (fatty acid-binding proteins); *de novo* synthesis: *ACACA* (acetyl-CoA carboxylase) and *FASN* (fatty acid synthase); fatty acid desaturation: SCD (stearoyl-CoA desaturase) and; esterification: *GPAT* (glycerol-3-phosphate acyltransferase), *AGPAT* (acylglycerol-3-phosphate acyltransferase), and *DGAT* (diacylglycerol acyltransferase). Finally, *mTOR* also can influence lipid metabolism by modulating the synthesis and activity of enzymes critical for fatty acid metabolism, linking energy availability to milk and lipid production in the mammary gland during lactation.

Figure 1 - Pathways and genes related to lactose and protein synthesis and lipid modulation that thiazolidinedione (TZD) can alter in mammary gland tissue.



Personal archive (2025). ACACA, Acetyl-CoA Carboxylase Alpha; AGPAT, 1-Acylglycerol-3-Phosphate O-Acyltransferase; AKT, Protein Kinase B; ATGL, Adipose Triglyceride Lipase; B4GALT, Beta-1,4-Galactosyltransferase; CD36, Cluster of Differentiation 36; CSN1S1, Casein Alpha S1; CSN2, Casein Beta; CSN3, Casein Kappa; DGAT, Diacylglycerol O-Acyltransferase; FABP, Fatty Acid-Binding Protein; FASN, Fatty Acid Synthase; GLUT1, Glucose Transporter 1; GPAT, Glycerol-3-Phosphate Acyltransferase; HSL, Hormone-Sensitive Lipase; LALBA, Alpha-Lactalbumin; mTOR, Mechanistic Target of Rapamycin; PPARG, Peroxisome Proliferator-Activated Receptor Gamma; SCD, Stearoyl-CoA Desaturase; STAT5, Signal Transducer and Activator of Transcription 5.

The TZD use in dairy cows has improved insulin sensitivity (KUSHIBIKI et al., 2001), altered plasma glucose dynamics, non-esterified fatty acids (NEFA) and beta-hydroxybutyrate, improved dry matter intake and body condition score (BCS) (SMITH et al., 2009; SCHOENBERG and OVERTON, 2011) and increased expression of lipogenic genes in adipose tissue of ovine fetuses at the end of pregnancy (MUHLHAUSLER et al., 2009). In lactating cows, activation of PPARG by TZD may have important implications in the control of energy homeostasis, particularly in the postpartum and early lactation periods, when insulin concentration is reduced and lipolysis is accentuated; and peak production, in which the demand for energy in epithelial cells for the synthesis of milk components is high (HOSSEINI et al., 2015).

However, studies that investigate the ways in which this substance acts on the metabolism of different species and, mainly, on its implications for the expression of mammary tissue genes are scarce.

3 NUTRITIONAL STRATEGIES TO IMPROVE THE GROWTH OF WEANED PIGLETS

Diet quality is a key factor in causing dietary stress and inducing oxidative stress in weaned piglets. Non-digestible protein sources when in an immature intestine, cause inflammation by the pathogenic bacteria and triggers an oxidative stress as described before. In addition, antinutritional factors from grains as soya, oxidate fat and mycotoxin present in piglets solid diet increase the intestine inflammation (ZHENG et al., 2021). Since stress is mainly responsible for the metabolic changes that affect the animal's performance during this stage, dietary strategies that mitigate these negative effects on piglet metabolism and promote growth and intestinal health are being explored.

Protein hydrolysis by chemical, microbial or enzymatic processes is known to increase grain quality, reducing anti-nutritional factors and ensuring greater protein digestion in the small intestine (ZHENG et al., 2021). In soybean, the main oilseed

used in the diet of pigs, besides to hydrolysis by enzymatic treatment, a fermented process using microorganisms increases the concentration of crude protein and amino acids compared to conventional soybean meal (KIM et al., 2010). In addition, adding food enzymes as carbohydrase (KIM et al., 2003), xylanase (CHEN et al.2020), protease (ZUO et al., 2015) and phytase (KIES et al., 2006) to the diet improve nutrients digestibility and reduces gut inflammation.

The addition of prebiotics to alter the microbiota by a substrate and probiotics by ingestion of beneficial microorganisms in the feed are used to modulate the microenvironment of intestine and exert benefits to the health of piglets (MARKOWIAK & SLIZEWSKA, 2018; DUARTE et al, 2020). Furthermore, bioactive compounds produced by fermentation process of probiotic microorganisms, called postbiotics, are recently used to modulate the intestinal immune status and health of nursery pigs (HOLANDA et al., 2020).

Plant-based additives such as phytobiotics and phytogenic also modulate the microbiota, improve feed palatability, and have anti-inflammatory and antioxidant properties in weaning piglets (ZHENG et al., 2021). Another exogenous source of bioactive molecules, the nucleotides, has been shown to alleviate the effects of weaning stress and improve feed efficiency (JANG et al. 2019). And organic acids as formic and propionic acids, also has been demonstrated anti-inflammatory responses (REN et al., 2019).

In a review, Hao et al. (2021) demonstrated nutritional modulation measures to mitigate oxidative stress in piglets and classified the most used supplements into four categories: 1) Amino acids, such as cysteine, arginine, and tryptophan; 2) Vitamins A, C and E; 3) Mineral elements, such as zinc, copper, manganese, selenium; and 4) Natural compounds, like curcumin and resveratrol.

In addition to the strategies applied in the solid diets, milk replacers are widely used in the transition from a liquid diet (sow's milk) to a solid one, helping in intestinal maturity by affecting enzymatic activity and morphology in weaning piglets (KELLY et al., 1991). Commercial milk substitutes basically consist of skimmed milk powder and whey, with the addition of vegetable components - such as vegetable starch and proteins from wheat and soya. However, as the small intestine of the piglet is not yet capable of digesting vegetable components, these substitutes can also lead to inflammatory processes and their consequences during the weaning phase (PLUSKE et al. 1997). In this sense, other dietary sources are being studied as alternatives. The

use of polar lipids from cow's milk fat globules, such as glycerophospholipids and sphingolipids, has been shown to have anti-inflammatory and protective effects in the gut (NORRIS et al. 2017).

3.1 POLAR LIPIDS

Polar lipids, or sphingolipids, account for less than 2% of the lipids in milk. They are amphiphilic lipids that have a hydrophilic head and a hydrophobic tail and are present in the membrane of fat globules (LOPEZ et al., 2019). It is believed that sphingolipids prevent globule coalescence, stabilize the emulsion and may be essential for the formation and maintenance of the membrane structure of milk fat globules (JENSEN, 2002).

Sphingolipids are composed of fatty acid chains with varied saturation, hydroxylation and glycosylation, which gives them metabolic properties. Derived from sphingosine, milk sphingolipids contain approximately 75% of sphingomyelin in their composition (main constituent of the myelin sheath; CHRISTIE et al., 1987), a phosphosphingolipid formed by the union of sphingosine with a fatty acid and choline. Plant-based products, such as soy, do not contain sphingomyelin.

The presence of sphingomyelin and ceramide in milk impart anticancer and anti-inflammatory properties to dairy products (NORRIS et al., 2017). Sphingomyelin together with phosphatidylserine (a glycerophospholipid) may also exert antimicrobial, antioxidant and neuroprotective effects (KIM et al., 2010) and be a dietary source of phosphocholine, promoting neonatal development (ZEISEL et al., 1986). Furthermore, sphingomyelin can reduce triglyceride accumulation in the liver and reduce circulating inflammatory cytokines, attenuating macrophage inflammatory responses following pro-inflammatory stimuli (NORRIS et al. 2017).

In a study with piglets from 2 to 30 days old, whose only food source was a milk replacer enriched with cow's milk fat globules and bioactive compounds, Berding et al. (2016) reported an 8% increase in the final weight of piglets, improvement in intestinal maturation and modulation of the intestinal microbiota. However, further studies are needed to understand the mechanisms related to the action of polar lipids in the metabolism of piglets under stress and the use of techniques such as omics may improve the specificity of the research.

4 OMICS APPROACHES IN ANIMAL NUTRITION

The term “omics” refers to a group of scientific fields that study molecules within biological systems on a comprehensive scale. Specialties explored by these fields include genomics, lipidomics, and microbiomics, which investigate genes, lipids, and microbial communities, respectively (SINGH et al., 2020). Omics approaches allow researchers to explore complex biological interactions holistically, providing deeper insights into how nutrients influence metabolism, health and performance (YATA et al., 2024). By uncovering these molecular processes, omics technologies are transforming animal nutrition and enabling more precise and efficient feeding strategies.

Nutrigenomics investigates how nutrients influence gene expression, revealing genetic pathways linked to traits such as growth, immunity, and feed efficiency. In swine nutrition, it has been used to develop diets that increase disease resistance (RASLAN et al. 2023), test new feed additives, and improve performance and meat quality (BANERJEE et al., 2015; MALGWI et al., 2022) through the targeted activation of specific genes. Lipidomics focuses on the study of lipids to assess how dietary fats from different sources affect animal performance (HOU et al., 2024) and how meat lipid composition can influence meat quality and promote health benefits for consumers (GAO et al. 2024). Meanwhile, microbiome studies the gut microbiota, discovering how interventions such as prebiotics, probiotics and even dietary components can improve digestion, nutrient absorption and immune function in pigs (DUARTE et al., 2020; HOU et al., 2024). Together, these omics tools provide a robust framework for advancing swine nutrition, improving productivity and the quality of animal products. Therefore, they are increasingly being applied in scientific research in this sector.

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HYPOTHESIS

GENERAL

Strategic nutritional modulation, analyzed through omics tools, positively alters the metabolic, lipid, and microbial profiles of weaned piglets and regulates gene expression associated with lipid, protein, and lactose metabolism in lactating sows, promoting improvements in animal health and productivity.

SPECIFICS

- TZD may stimulate mechanisms that regulate transcription via *mTOR*, altering protein and lactose synthesis in milk, as well as altering lipid regulation in the mammary gland and adipose tissue of lactating sows.
- The polar lipids in the diet and milk replacers would result in extensive changes in the plasma lipidomic profile of growing piglets, which can be associated with modulation of inflammatory markers during the first 7 days after weaning.
- Lipids from a diet composed of sphingolipids would have a different impact on the composition of the intestinal microbiota compared to a soy-based diet.
- Changes in the composition of the microbiota would be associated with the modulation of several types of lipids, including endocannabinoidome (eCBome) lipids and those related to inflammation and animal performance.

OBJECTIVES

GENERAL

To investigate the effects of nutritional modulation in swine using omics approaches, focusing on lipidomics and microbiome analyses in weaned piglets and nutrigenomics in lactating sows, aiming to understand the underlying molecular mechanisms impacting health, productivity, and milk quality.

SPECIFICS

- To evaluate the effect of thiazolidinedione (TZD) supplementation on insulin activity and the *mTOR* pathway in lactating sows.
- To analyze the gene expression related to lipogenesis, protein synthesis, and lactose production in response to TZD supplementation.
- To investigate the changes in milk composition of sows under nutritional treatment with TZD.
- To evaluate the impact of a modified by-product of cheese-making rich in polar lipids on the plasma lipidomic profiles of piglets during the weaning period.
- To study the influence of the dietary fatty acid profile on plasma lipid composition.
- To evaluate the use of a dairy processing byproduct rich in polar lipids in the microbiome and plasma lipid mediators of piglets during the weaning period.

CHAPTER II

THIAZOLIDINEDIONE-INDUCED ALTERATIONS IN LIPID AND PROTEIN-RELATED GENE EXPRESSION IN LACTATING SOWS

THIAZOLIDINEDIONE-INDUCED ALTERATIONS IN LIPID AND PROTEIN-RELATED GENE EXPRESSION IN LACTATING SOWS

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ABSTRACT

Thiazolidinedione (TZD) is a chemical agonist of transcription factor peroxisome proliferator-activated receptor gamma (*PPARG*) that can stimulate insulin cellular signaling by increasing protein synthesis and altering lipogenesis in the mammary gland and adipose tissue. The objective was to evaluate the effect of TZD on insulin and mammalian target of rapamycin (*mTOR*) activity, lipogenic, protein and lactose gene expression, and milk composition of lactating sows. Forty lactating multiparous sows were randomly assigned to one of two treatments for 15 days (from day 13 to day 28 of lactation): 1) Control (no TZD added); 2) 8 mg/kg BW of TZD mixed with feed. TZD did not affect insulin, glucose and non-esterified fatty acids (NEFA) in the plasma. There was no effect on milk composition, however, TZD reduced saturated fatty acids (SFA; P = 0.02) and increased monounsaturated fatty acids (MUFA; P = 0.03) by 11.3 and 13.7% in the milk FA profile, respectively. In addition, there was an increase of 20.1% in the expression of *AGPAT6* (P = 0.001) and trends of *DGAT1* (P = 0.08) and *SCD* (P = 0.06) in the mammary gland of TZD-treated sows. Lipogenic genes in adipose tissue were not affected. Among the genes related to protein synthesis in mammary gland tissue, *CSN3* increased by 42.7% (P = 0.02) in TZD compared to Control. In summary, TZD increases the expression of genes related to lipid and protein synthesis in the mammary gland and alters SFA and MUFA in the milk profile of lactating sows.

KEYWORDS: Milk fat; Milk protein; Insulin; Lactation in sows.

1 INTRODUCTION

The productivity of an adult swine is directly related to its performance as a piglet in the maternity ward and to the sow's ability to meet the demands of the litter during pregnancy and lactation (KING, 2000). Since milk is the main source of nutrients for the piglet until weaning, improving its composition and quantity through the sow's diet can be an effective strategy in production systems that have insufficient milk production as a performance limiting factor from the sow to the number of piglets born at each farrowing or that aim to increase the weight of the piglets at weaning.

Although the amount of milk protein is mainly determined by the animal's genetics, it can be sensitive to diet composition (PEDERSEN, 2018). In addition, some metabolic modifiers may influence insulin regulation by interfering with the process of protein biosynthesis through peroxisome proliferator-activated receptors gamma (*PPARG*; REYNOLDS; GOLDBERG, 2006). Thiazolidinedione (TZD) is a *PPARG* chemical agonist that can stimulate cell insulin signaling by increasing protein synthesis via the mammalian target protein of rapamycin (*mTOR*) as well as acting on the family of transcription factors responsible for lipid homeostasis – the sterol regulatory element binding protein (*SREBP1*), altering the expression of genes related to the synthesis, uptake, and esterification of fatty acids in adipose and mammary gland tissue (BIONAZ et al., 2013). Studies in ruminants have shown that TZD is effective in postpartum energy regulation and has potential to improve milk composition (HOSSEINI et al., 2015). In pigs, TZD has been shown to alter fat metabolism and meat quality, improving marbling score and intramuscular fat content (CHEN et al., 2013; JIN et al., 2018). However, the effects of TZD may vary according to the animal species and tissue tested and no reports on the effect of TZD on sow milk composition have been found.

We hypothesized that TZD may stimulate mechanisms that regulate transcription via *mTOR*, altering protein and lactose synthesis in milk, as well as altering lipid regulation in the mammary gland and adipose tissue of lactating sows. To investigate these responses, we evaluated the TZD agonist effects on milk composition, insulin activity on milk protein and lactose synthesis, *mTOR* activity and lipid regulation of lactating sows.

2 MATERIAL AND METHODS

2.1 ANIMALS, DESIGN AND TREATMENTS

All procedures were approved by the Santa Catarina State University Ethical Committee, protocol n. 9630220224, and were performed at a commercial farm in Chapecó, SC (27° 06' 17" S 52° 36' 51" W). Forty multiparous sows from a commercial genotype in their 2^{nd} – 6^{th} parities and weighting (BW) 250 ±15 kg were randomly assigned to one of the following treatments: 1) Control, without TZD added to the diet or; 2) 8 mg/kg of BW of TZD mixed into the diet. The amount of TZD mixed into the feed was based on the studies with cows from Arévalo-Turrubiarte et al. (2012) and Yousefi et al.(2015).

2.2 MANAGEMENT, FEEDING AND EXPERIMENTAL PERIOD

The experimental period was 15 d and TZD feeding started on day 13 of lactation and was maintained till day 28 of lactation. The sows were moved into farrowing rooms after 110d of gestation and were housed individually in pens (2.2×1.6 m) with slatted floors and controlled temperature and relative humidity. The experimental diets were formulated according to the company to which the farm was integrated to meet the recommended nutrients required by the animals and contained ground corn (70%), soybean meal (21.4%), deactivated soybean (2.0%), commercial vitamin/mineral mix (5%), sugar (1.4%), adsorbent (0.2%), acidifier (0.1%; BioSyn AMA®, Brazil), and 50g of symbiotic bioactive complex additive (BioSyn AMA®, Brazil). The nutritional composition of diets contained, on average, 17% of crude protein, 4% of ether extract, 1.4% of calcium, 0.61% of phosphorus, 2.4% of crude fiber and 7.4% of ash. Sows were fed three times per d and received 7.0 ± 0.5 kg/d (as fed basis) of the ration. The treatment was provided in the first meal, and the orts were collected and weighed. Water access was *ad libitum.*

The number of piglets in each litter was adjusted (twelve piglets per sow) by cross-fostering piglets within 24 h of birth. In the first 5 d after birth, the litters were subjected to normal management procedures, including cutting of milk teeth, tail docking, ear notching, iron shots and castration of males. At two weeks old, the piglets began to receive supplemental dry feed for adaptation to a solid diet. The litters from

both treatments received the same ration, and the intake was approximately 0.80 kg (as fed) during this period.

2.3 GLUCOSE, INSULIN AND NEFA ANALYSIS

Blood samples were collected from the forty sows on d 13 and 28 of lactation for glucose analysis using the ReliOn device (blood glucose monitoring system). In addition, blood samples (4 ml) from the forty sows were also collected on d 28 of lactation in tubes containing EDTA and centrifuged at 3,000 rpm for 10 minutes at 5°C. The serum was separated and stored at -20°C for insulin and non-esterified fatty acids (NEFA) analysis.

Insulin analysis was performed by the Immulite® 1000 chemiluminescent immunoassay, according to the internal protocol of the laboratory (Axys Análises Diagnóstico Veterinário e Consultoria LTDA, Chapecó, SC, Brazil). NEFA analysis was performed by the Trinder enzymatic method using the NEFA VET kit (Bio Técnica Ind. Com. LTDA, MG, Brazil) and following the manufacturer's protocol. The determination was performed by spectrophotometry using an automatic biochemical analyzer (SX 160, Sinnowa Healthcare®).

2.4 MILK COMPOSITION AND MILK FATTY ACID PROFILE ANALYSIS

Milk samples were collected from 6 sows in each treatment on d 13, 21 and 28 of lactation. Approximately 50 ml of milk was obtained by manual stripping after intravenous injection of 0.5 ml of oxytocin (Ocitovet©; Ceva Santé Animal). The samples were stored at 4°C with a preservative (Bronopol tablet; D & F Control Systems Inc.) and sent to composition and fatty acid profile analysis. Milk fat, protein, lactose and total solids were determined by infrared analysis.

Milk fatty acid profile was determined on milk collected on day 28 of lactation. Milk fat cake was obtained by centrifuging refrigerated milk at 3000rpm for 15min at 4°C. Approximately 50 mg was extracted then methylated following the methodology described by Hara et al. (1978) and Christi e (1982).

2.5 MAMMARY AND ADIPOSE TISSUE BIOPSIES

On the last day of the experimental period (d 28 of lactation), biopsies of the mammary gland and adipose tissue were performed. A tranquilizer was administered (2ml/sow intramuscular and 6ml/sow intravenous of Destress injectable; Des-Far Laboratories LDTA) to immobilize the animals, and lidocaine hydrochloride subdermal (2 ml/sow) was then administered above the incision site. A coaxial needle with a trocar was introduced to the first or second thoracic mammary glands. The biopsy was collected using a Bard Max-Core Disposable Core Biopsy Instrument (Bard Biopsy Systems). Briefly, a 16-gauge biopsy needle was partially inserted through the coaxial needle and two tissue samples (approximately 35mg tissue/biopsy) were collected. These were inspected to verify tissue homogeneity, rinsed with sterile saline solution and were immediately stored into aluminum foil in liquid N_2 until RNA extraction. The biopsy procedure resulted in minimal bleeding and no intra-mammary infections were observed.

The adipose tissue biopsy was taken from the tail head region immediately cranial and lateral to the last lumbar vertebra (dorsal subcutaneous depot). Prior to the biopsy, site asepsis was performed and lidocaine hydrochloride subdermal was administered in a circular pattern surrounding the incision site (2ml/sow). A small incision was made in the skin, and adipose tissue was dissected. Two samples of adipose tissue (approximately 100mg) from the same site were obtained, rinsed with sterile saline solution, placed in aluminum foil and frozen in liquid N_2 until RNA extraction. The incision was closed with number 1 Nylon using a blanket stitch. After biopsies, an anti-inflammatory was administered (flunixinmeglumine;1.1mg/kg of BW).

2.6 RNA EXTRACTION, SYNTHESIS OF COMPLEMENTARY DNA AND QUANTITATIVE REAL-TIME RT PCR

Total RNA extraction, synthesis of complementary DNA and quantitative real-time PCR (RT-qPCR) were all carried out according to the methodology of Sandri et al. (2018). Briefly, total RNA was extracted from both mammary and adipose tissue samples using the RNeasy Lipid Tissue Mini Kit (Qiagen Sciences) with on-column DNAse treatment (On-Column DNase I Digestion Set; Sigma-Aldrich). The RNA concentration was measured using a spectrophotometer (NanoDrop ND 2000;

NanoDrop Technologies) and, using the same spectrophotometer, the quality was evaluated by the $A_{260/280}$ ratio, which was approximately 2.03 (SE 0.01). Total RNA was transcribed to complementary DNA using the Go Script™ Reverse Transcription Mix (Promega Corporation) with random primers. PCR amplification was performed in triplicates in a 96-well reaction plate (Micro Amp™; Applied Biosystems) with 15 µl volume reaction with 30 ng of complementary DNA and 7·5µl Go Taqq PCR Master Mix (Promega) in an Applied Biosystems ABI 7500 Real-Time PCR" device (Applied BioSystems, USA) and the data were analyzed with the 7500 software 2.3 (Applied BioSystems, USA). All primers used were previously validated for the formation of a single product by melting curve and amplification efficiency. Each sample was run using seven-point standard curves with a 'pool' of complementary DNA from the mammary or adipose tissues with serial dilutions (100, 50, 25, 12.5, 6.25, 3.125 and 1.5625%). Subsequently, a regression equation was generated by plotting the cycle threshold values from RT-qPCR against the log of each value from the standard curve. The slope of the equation was used to determine the efficiency of the reaction.

2.7 PRIMER DESIGN

Gene sequences for primer designs were obtained from the gene bank of the National Center for Biotechnology Information (NCBI, USA). All primers were designed by the Prime-BLAST tool of NCBI, synthesized at Invitrogen™ and were tested for their efficiency before use. Expression of the following genes was measured: casein-αS1 (*CSN1S1*), casein-β (*CSN2*), casein-κ (*CSN3*), α-lactalbumin (*LALBA*), beta-1,4-galactosyltransferase (*B4GALT*), acetyl-CoA carboxylase-α (*ACACAα*), fatty acid synthase (*FASN*), stearoyl-CoA desaturase 1(*SCD1*), lipoprotein lipase (*LPL*), fatty acid binding protein 3 (*FABP3*), acyl glycerol phosphate acyltransferase 6 (*AGPAT6*), diacylglycerol acyltransferase1 (*DGAT1*), transcription factors peroxisome proliferator-activated receptor gamma (*PPARG*) and mammalian target protein of rapamycin (*mTOR*). The primer sequences of the evaluated genes are listed in Table 1.

Table 1 - Swine primers used in the quantitative real-time RT PCR (RT-qPCR) analysis for mammary gland and adipose tissue

Symbols	Forward (F) and Reverse (R) primer*	Mammary gland		Adipose tissue	
		R^2	Efficiency	R^2	Efficiency
RPS18	F: CTGGCCAACGGTCTGGATAA	99.7	90.0	99.9	90.0
	R: GGACACGCAGTCCCCAGAA				
ACTB	F: TCGCCGACAGGATGCAGAA	99.9	90.3	99.8	98.5
	R: CCGATCCACACGGAGTACTTG				
ACACAα	F: CCTGCCCTAGCTTTCCAGTTAGAG	99.1	93.5	99.3	110.0
	R: TGGCTGCCCCAAGGTACA				
FASN	F: GAACCTGGAGGAGTTCTGGGC	99.5	93.3	99.7	94.5
	R: ATCGTGTTCGCCTGCTTGGA				
SCD1	F: GTGACCCTGGGCAAGTCATTTA	99.8	91.1	99.7	100.4
	R: ACGCCTCAAAACTGCCCTTT				
LPL	F: AGATGTGGACCAGCTCGTGAA	99.9	90.1	99.9	101.3
	R: GCACCGGTAGGCCTTACTAGGA				
FABP3	F: CAAGCTGGGAGTGGAGTTTGAT	99.8	98.1	99.7	102.9
	R: CCACTTCTGCACGTGGACAA				
AGPAT6	F: CTCCCCACGTCTGGTTCGAA	99.9	91.7	99.4	97.3
	R: AGGATGGGCAGCTTGCTTTT				
DGAT1	F: GCCTGCAGGATTCTTTGTTCAG	99.9	91.5	99.9	80.1
	R: AGCCGTGCATTGCTCAAGAC				
PPARG	F: AAGAGCCTTCCAACTCCCTCA	99.9	83.7	99.3	102.7
	R: CCGGAAGAAACCCTTGCAT				
CSN1S1	F: GCCATGAGCAAAGGGGATCT	99.8	106.1	-	-
	R: AGGCTCTCCCTGTTGGGTAT				
CSN2	F: GCCATGAAGCTCCTCATCCT	99.9	93.7	-	-
	R: AGGCTTTCCACAGTCTCACC				
CSN3	F: TTTGGGTGCAGAGGAGCAAA	99.9	90.8	-	-
	R: AGCTACAACTGGCCTTGCAT				
LALBA	F: ATGTGAACACCCGCTGTCTT	99.9	91.9	-	-
	R: GCACCAGTCACGCATCTCTA				
B4GALT1	F: GCTCCTCAACGTCGGCTTTA	99.9	95.6	-	-
	R: GGCATTGTGGTCATTCATTGG				
mTOR	F: GCCCCCGATCGTGAAGTTAT	99.6	97.4	-	-
	R: TGAAATCCAGAGACTCCGTCAGA				

RPS18, ribosomal protein S18; *ACTB*, β-actin; *ACACAα*, acetyl-CoA carboxylase-α; *FASN*, fatty acid synthase; *SCD1*, stearoyl-CoA desaturase 1; *LPL*, lipoprotein lipase; *FABP3*, fatty acid binding protein 3; *AGPAT6*, acyl glycerol phosphate acyltransferase 6; *DGAT1*, diacylglycerol acyltransferase 1;

PPARG, transcription factors peroxisome proliferator-activated receptor gamma; *CSN1S1*, casein-αS1; *CSN2*, casein-β; *CSN3*, casein-κ; *LALBA*, α-lactalbumin; *B4GALT1*, β-1,4 galactosyltransferase 1; *mTOR*, mammalian target protein of rapamycin. *Primers are reported as 5' to 3' sequence.

2.8 STATISTICAL ANALYSIS

Data were analyzed using the MIXED procedure of the SAS statistical program(2017). For the analysis of production and concentration of milk components diet intake was considered as a covariate, treatments as a fixed effect and animals as a random effect. For the analysis of gene expression, the geometric measure of the "housekeeping" genes ribosomal protein *S18* (*RPS18*) and Actin-beta (*β-Actin*) was used as a covariate in the model.

Data points with Studentized residual outside of ± 2.5 were considered outliers and were excluded from analysis. Least-squares means (LSMEANS) were used to compare treatments and a significant difference was considered when $P < 0.05$ and a trend at $P < 0.10$.

3 RESULTS

3.1 DIET INTAKE, PLASMA PARAMETERS AND MILK COMPOSITION

Diet intake, insulin, glucose and NEFA in plasma and milk composition results are present in Table 2. These variables were not affected by TZD ($P > 0.05$).

Table 2 - Effect of thiazolidinedione (TZD) on diet intake, insulin, glucose and non-esterified fatty acids (NEFA) in the plasma and milk composition of lactating sows

	Treatments			
Variable	Control	TZD	SEM	*P**
Intake, kg	5.33	5.14	0.09	0.14
Plasma				
Insulin, mcU/mL	<0.5	<0.5	-	-
Glucose, mg/dL	71.75	73.34	1.57	0.48
NEFA, mmol/L	0.18	0.14	0.03	0.40
Milk composition				
Fat, %	5.52	5.60	0.31	0.85
Protein, %	5.04	5.15	0.06	0.24
Lactose, %	5.92	5.78	0.08	0.25
Casein, %	4.23	4.27	0.06	0.64
Total solids, %	17.42	17.46	0.31	0.93

*Overall effect of treatment.

3.2 MILK FATTY ACID PROFILE ANALYSIS

Milk fatty acid composition is shown in Table 3. The main FA found in sows' milk were 16:0, 16:1, 18:1 and 18:2. TZD reduced concentrations of 8:0 (P = 0.02), 10:0 (P = 0.04) and 16:0 (P = 0.02) by 32.0, 39.9 and 15.8%, respectively. While 18:1 *trans* (P = 0.02), 18:1 *cis*-9 (P = 0.04) and 18:1 *cis*-13 (P = 0.01) were increased by 64.9, 35.3 and 88.5%, respectively. The sum of saturated fatty acids (SFA) decreased by 11.3% (P = 0.02), while the sum of monounsaturated fatty acids (MUFA) increased by 13.7% (P = 0.03) in TZD treatment.

Table 3 - Effect of thiazolidinedione (TZD) on milk FA profile of lactating sows

	Treatments			
FA, % of total FA	Control	TZD	SEM	*P**
6:0	0.04	0.04	0.02	0.77
8:0	0.06	0.04	0.01	0.02
10:0	0.35	0.21	0.06	0.04
10:1	0.06	0.04	0.01	0.13
11:0	0.01	0.01	0.00	0.08
12:0	0.29	0.28	0.08	0.95
13:0 iso	0.13	0.19	0.07	0.46
14:0	4.73	3.99	0.71	0.33
14:1 *cis*-9	0.46	0.32	0.12	0.30
15:0	0.12	0.09	0.02	0.19
15:0 iso	0.08	0.11	0.03	0.44
16:0	35.88	30.21	1.87	0.02
16:0 iso	0.01	0.02	0.01	0.52
16:1 *cis*-9	13.22	9.27	2.17	0.11
17:0	0.15	0.17	0.02	0.53
17:0 iso	0.07	0.06	0.01	0.49
17:1	0.19	0.20	0.04	0.73
18:0	2.47	4.02	0.92	0.13
18:1 *trans*	0.10	0.16	0.02	0.02
18:1 *cis*-9	24.68	33.39	3.46	0.04
18:1 *cis*-11	1.89	2.59	0.32	0.06
18:1 *cis*-12	0.09	0.14	0.03	0.12
18:1 *cis*-13	0.05	0.10	0.01	0.01
18:2 *cis*-9 *cis*-12	12.99	12.16	0.59	0.20
18:2 *cis*-9 *trans*-11	0.07	0.07	0.04	0.84
18:3 *n*-6	0.08	0.09	0.02	0.76
18:3 *n*-3	0.76	0.75	0.05	0.76
20:0	0.06	0.07	0.02	0.62

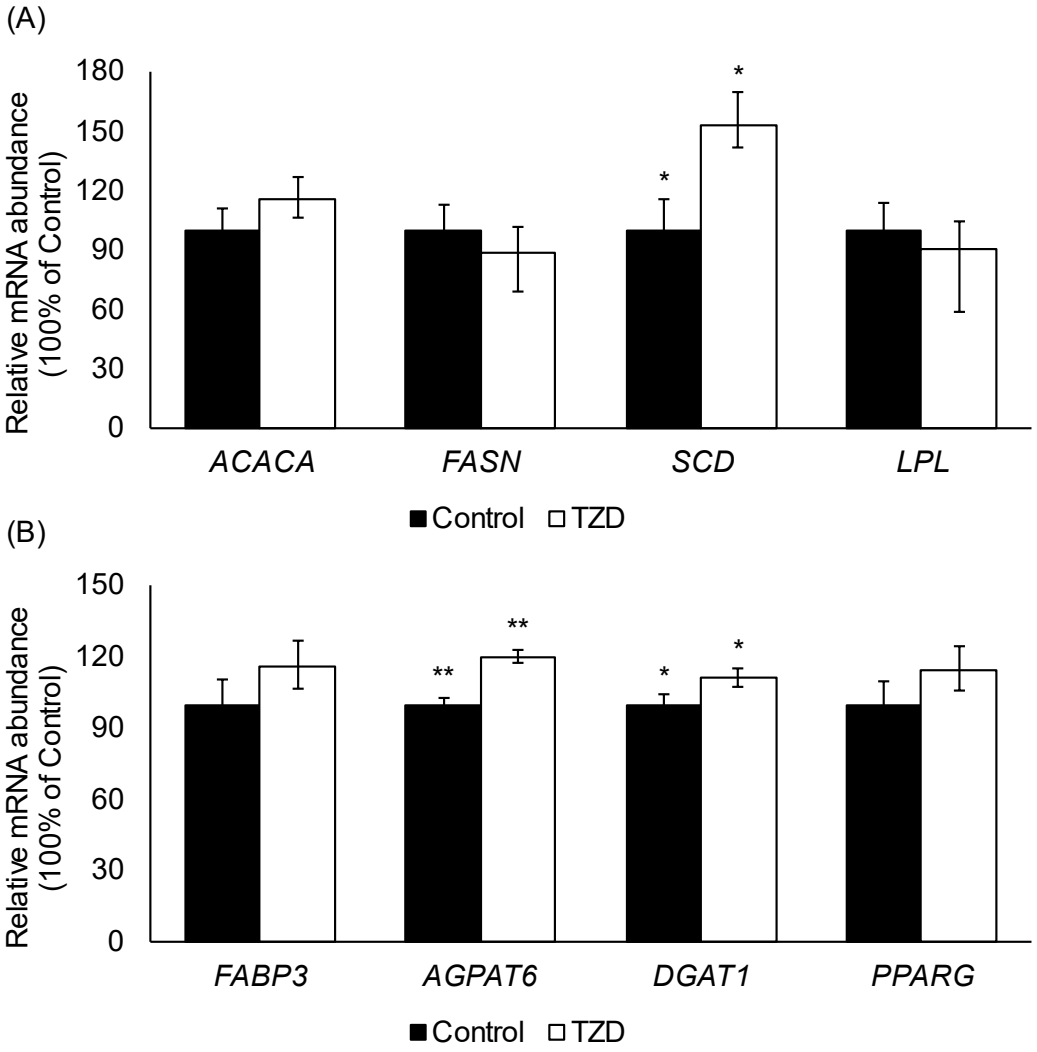
20:2	0.16	0.27	0.07	0.12
20:3 *n*-3	0.02	0.03	0.01	0.34
20:4 *n*-6	0.40	0.43	0.04	0.45
20:5 *n*-3	0.01	0.02	0.00	0.18
22:0	0.05	0.07	0.02	0.40
22:1 *n*-9	0.04	0.05	0.01	0.21
22:2	0.02	0.12	0.10	0.33
22:5	0.06	0.07	0.01	0.71
∑ SFA†	43.08	38.21	1.69	0.02
∑ MUFA†	39.79	45.24	2.03	0.03
∑ PUFA†	12.99	12.51	0.63	0.46

*Overall effect of treatment.
†∑SFA, ∑MUFA, ∑PUFA: sum of SFA, MUFA and PUFA, respectively.

3.3 EXPRESSION OF LIPOGENIC GENES IN MAMMARY GLAND AND ADIPOSE TISSUE

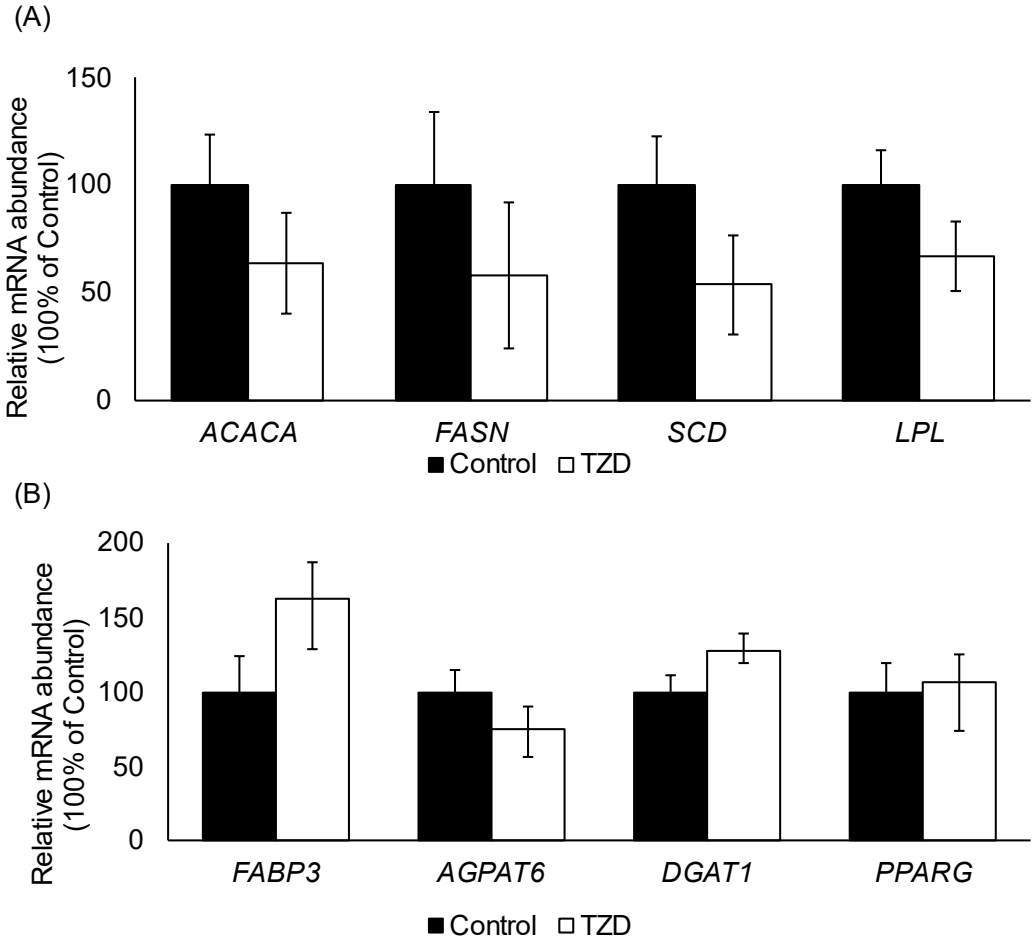
In the mammary gland, TZD increased the expression of the TG synthesis gene *AGPAT6* by 20.1% ($P = 0.001$; Fig.1B) while there was a trend of increase in *SCD* ($P = 0.06$; Fig.1A) and *DGAT1* ($P = 0.08$; Fig. 1B) and There was no effect of TZD on adipose tissue gene expression ($P > 0.05$; Fig.2).

Figure 1 - Acetyl-CoA carboxylase-α (*ACACA*), fatty acid synthase (*FASN*), stearoyl-CoA desaturase 1 (*SCD1*), lipoprotein lipase (*LPL*) (A), fatty acid binding protein 3 (*FABP3*), acyl glycerol phosphate acyltransferase 6 (*AGPAT6)*, diacylglycerol acyltransferase 1 (*DGAT1*) and transcription factors peroxisome proliferator-activated receptor gamma (*PPARG*) (B) gene expression in the mammary gland of sows supplemented with thiazolidinedione (TZD) compared with control



Values are means with their standard errors. Significant differences ($P < 0{\cdot}05$) are denotated with ** while trends ($P < 0{\cdot}10$) are denotated with*.

Figure 2 **-** Acetyl-CoA carboxylase-α (*ACACA*), fatty acid synthase (*FASN*), stearoyl-CoA desaturase 1 (*SCD1*), lipoprotein lipase (*LPL*) (A), fatty acid binding protein 3 (*FABP3*), acyl glycerol phosphate acyltransferase 6 (*AGPAT6*), diacylglycerol acyltransferase 1 (*DGAT1*) and transcription factors peroxisome proliferator-activated receptor gamma (*PPARG*) (B) gene expression in the adipose tissue of sows supplemented with thiazolidinedione (TZD) compared with control

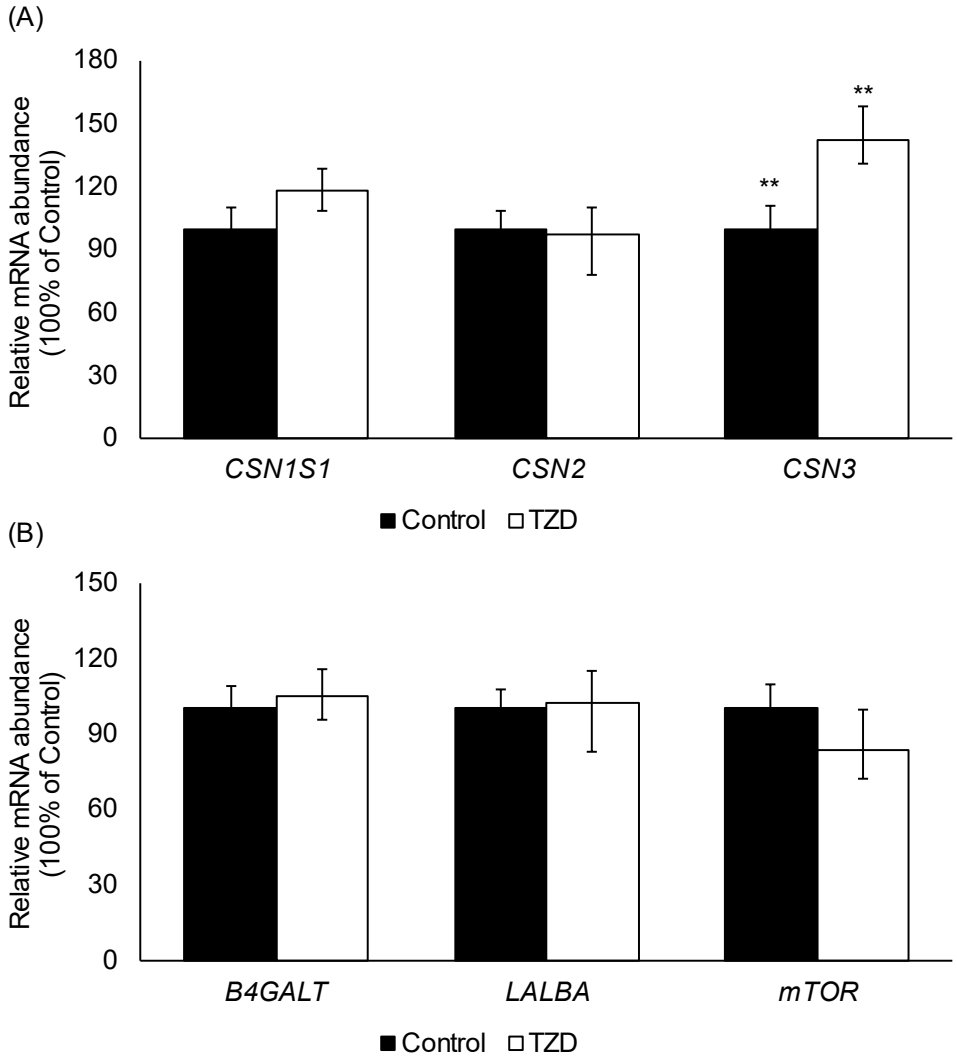


Values are means with their standard errors. Significant differences ($P < 0{\cdot}05$) are denotated with ** while trends ($P < 0{\cdot}10$) are denotated with*.

3.4 EXPRESSION OF GENES INVOLVED IN PROTEIN AND LACTOSE SYNTHESIS IN MAMMARY GLAND

There was an increase in *CSN3* expression of 42.7% in TZD treatment compared to control ($P = 0.02$; Fig.3A). While other caseins, lactalbumin and *mTOR* were not affected ($P > 0.05$).

Figure 3. Casein-αS1 (*CSN1S1*), casein-β (*CSN2*), casein-κ (*CSN3*) (A), β-1,4 galactosyltransferase 1 (*B4GALT*), α-lactalbumin (*LALBA*) and, mammalian target protein of rapamycin (*mTOR*) (B) gene expression in the mammary gland of sows supplemented with thiazolidinedione (TZD), compared with control



Values are means with their standard errors. Significant differences ($P < 0{\cdot}05$) are denotated with ** while trends ($P < 0{\cdot}10$) are denotated with*.

4 DISCUSSION

TZD is a molecule used to treat type 2 diabetes in humans. The dose of the drug may vary depending on the type of TZD (i.e. pioglitazone, rosiglitazone, 2,4-thiazolidinedione) and the route of administration. In animal experiments, the dose range varies from 1 to 15 mg/kg, usually by intravenous (IV) administration to ensure

greater absorption of TZD by the tissues (CHEN et al., 2013; JIN et al., 2018; KUDA et al., 2009; MIRZAIE et al., 2022; SARRAF et al., 2012; SMITH; BUTLER; OVERTON, 2009). In the present study, we offered TZD orally, mixed with the feed, to avoid possible stressful effects that intravenous manipulation may cause. We chose the dose of 8 mg/kg of TZD based on studies with cattle that demonstrated improvement in energy efficiency (ARÉVALO-TURRUBIARTE et al., 2012) and insulin sensitization (YOUSEFI et al., 2015) receiving the same dose of TZD orally.

TZD was expected to upregulate insulin, increasing intracellular glucose concentration, especially in mammary tissue, given the energy expenditure required for milk production in lactating sows. From this, effects on lipid and protein metabolism could be observed in milk. Intravenous administration of 4 mg/kg 2,4-TZD added to a higher-energy diet in non-pregnant and non-lactating cows was able to increase blood concentrations of glucose, insulin, and beta-hydroxybutyrate (BHBA), while NEFA and adiponectin concentrations remained unchanged (HOSSEINI et al., 2015). Similarly, 6 mg/kg pioglitazone supplemented in the diet increased glucose, insulin, BHBA concentrations and reduced negative energy balance in transition dairy cows (MIRZAIE et al., 2022). In pigs, plasma glucose was not affected by the administration of 1 mg/kg IV pioglitazone or rosiglitazone (SARRAF et al., 2012) or 15 mg/kg TZD supplemented in the diet (CHEN et al., 2013). Chen et al.(2013) further suggest that TZD influences serum glucose concentrations only when glucose metabolism is abnormal, having no effect in healthy animals. In the present study, circulating glucose and insulin metabolism, as well as NEFA, were not altered. Furthermore, the test used for insulin analysis was not able to detect concentrations below 0.5 mcU/mL.

Milk composition also did not show any change with the addition of TZD to the sows' diet, and the effects of this molecule on ruminants are controversial. Prepartum cows that received TZD at a dose of 4 mg/kg reduced fat percentage and milk yield corrected for fat percentage, while improving body condition score postpartum (SMITH; BUTLER; OVERTON, 2009). In sheep that received 4 mg/kg of intravenous TZD, there was an increase in the percentage of protein and fat in the milk (SANDRI et al., 2018). We did not observe any effect on the composition of sow's milk. It is important to emphasize that unlike ruminants, sows are not used to milking management and the management of separating piglets before collection, oxytocin injection to release milk

and milking manually generates a high level of stress in these animals. Increased cortisol reduces the effect of oxytocin and pauses milk release (WELLNITZ; BRUCKMAIER, 2001). Furthermore, the solid fraction of milk is concentrated at the end of milking and for a reliable analysis it is necessary to empty the mammary gland. Based on these facts, we report the difficulty in handling as a factor that influenced milk collection and possibly interfered with its actual composition. However, there were effects at the cellular level when we measured gene expression and fatty acid composition of milk, which were independent of the amount of milk collected.

The upregulation of insulin responsive to the effect of TZD is related to signaling through *PPARG*. The transcription factor *PPARG* promotes the expression of genes related to lipid metabolism and can also regulate protein synthesis through the *mTOR* pathway (BIONAZ et al., 2013). As in insulin sensitivity results, TZD did not alter the mRNA abundance of *PPARG* and *mTOR* in mammary tissue. Sandri et al.(2018) found an increase in the protein content of ewes milk related to the increased abundance of alpha, beta and kappa casein and *PPARG* mRNA abundance; however, the same authors reported a reduction in *mTOR* expression, signaling a possible alternative regulatory pathway. In our study, TZD increased only the mRNA abundance of *CSN3* - a gene related to kappa casein synthesis. Kappa casein plays an important role in the stabilization of casein micelles in milk. A higher concentration of kappa casein in milk allows the formation of a denser and more consistent curd in the gastric environment, which slows the rate of digestion and absorption (FITZPATRICK et al., 2024; HORSTMAN; HUPPERTZ, 2023). Slower gastric emptying may be beneficial by providing a gradual release of nutrients, resulting in greater absorption of amino acids into the blood of neonates (HUPPERTZ; CHIA, 2021). However, piglet performance was not the focus of the present research.

The *AGPAT6* and *DGAT1* genes increased by TZD in mammary gland tissue are involved in complementary and sequential pathways in lipid metabolism, especially in the context of triacylglycerol (TG) synthesis. *AGPAT6* catalyzes the conversion of lysophosphatidic acid to phosphatidic acid, and phosphatidic acid is subsequently converted to diacylglycerol (DAG), while *DGAT1* converts DAG and acyl-coenzyme A (CoA) to TG (COLEMAN, 2004). The increase in the expression of these genes without effect on *PPARG* suggests an activation of other lipogenic transcription factors (e.g.,

SREBP1, ChREBP; WANG et al., 2015) or a specific selection of target genes, when an increased demand for TG in milk directs the activation of genes involved in the final steps of lipogenesis, without altering upstream genes.

TZD also influenced enzymatic activity or metabolic flux in mammary gland tissue, favoring the conversion of SFA to MUFA. The enzyme related to this activity is *SCD*, which introduces a *cis* double bond between carbons 9 and 10 in a wide range of FAs, especially the conversion of stearic acid (18:0) to oleic acid (18:1 *cis*-9) and palmitic acid (16:0) to palmitoleic acid (16:1 *cis*-9; NTAMBI; MIYAZAKI, 2004). The abundance of *SCD* mRNA showed a tendency to increase in the TZD treatment, which explains the increase in the concentration of the sum of MUFAs and 18:1 cis-9 in the milk composition of sows from the same group. Similarly, Hosseini et al.(2015) observed an increase in the mRNA expression of the lipogenic enzymes *SCD* and *DGAT2* in one week after TZD administration with a decrease in two weeks after TZD administration in the subcutaneous adipose tissue of cows, further demonstrating a variation over time in the expression of these genes under TZD effect.

Furthermore, the increase in 18:1 *trans* and 18:1 *cis*-13 fatty acids after TZD treatment suggests hepatic or adipose tissue metabolism with mobilization to mammary gland tissue. However, TZD did not alter genes related to lipid regulation in adipose tissue, nor the abundance of genes related to transport in mammary gland tissue. Likewise, there was no treatment effect on the expression of genes related to de novo synthesis in the mammary gland that would justify the reduction in SFA concentration in the milk profile. Despite this, Hosseini et al. (2015) discussed possible variations in the expression of lipogenic genes over time under the effect of TZD, where there was a peak of expression and then an adaptation of metabolism followed by a reduction in expression in a 15-day interval. This leads us to suggest a possible occurrence of effects on the milk FA profile remaining from a peak of gene expression that occurred prior to tissue collection for expression analysis.

In addition to the variations in gene expression over time, Arévalo-Turubiarte et al. (2012) showed a reduction in *PPAR* expression in the liver, an increase in muscle, and no effect on subcutaneous adipose tissue in cattle. Taking these results together, there is a variation in the response of TZD to the type of tissue, as well as to the time of administration. Further studies are needed to clarify these effects in lactating sows.

5 CONCLUSION

TZD treatment in lactating sows increased the expression of *AGPAT6, DGAT, SCD* and *CSN3* in mammary tissue, which may improve pathways related to triacylglycerol, unsaturation rate and kappa-casein synthesis, respectively. TZD reduced SFA (8:0, 10:0, and 16:0) and increased MUFA (18:1 *trans*, 18:1 *cis*-9, and 18:1 *cis*-13) in milk, resulting in a more unsaturated fatty acid profile. These effects occurred without changes in plasma parameters, general milk composition, or *PPARG* and *mTOR* expression, suggesting selective modulation of mammary metabolic pathways.

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DECLARATION OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHORSHIP CONTRIBUTION

R.L., E.C.S. and D.E.O. designed the research. W.C. and E.M.T. provided financial assistance and animals for the experiment. R.L. and E.C.S. conducted the animal experiment. R.L., J.V.C., C.G.P., R.H. and D.P.D.L. conducted laboratory analysis. R.L. wrote the paper and analyzed the data analysis with guidance from D.E.O. All authors read and approved the final manuscript.

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Thiazolidinedione-induced alterations in lipid and protein-related gene expression in lactating sows

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Keywords:	Milk fat, Milk protein, Insulin, Lactation in sows
Subject Category:	Molecular Nutrition
Abstract:	Thiazolidinedione (TZD) is a chemical agonist of transcription factor peroxisome proliferator-activated receptor gamma (PPARG) that can stimulate insulin cellular signaling by increasing protein synthesis and altering lipogenesis in the mammary gland and adipose tissue. The objective was to evaluate the effect of TZD on insulin and mammalian target of rapamycin (mTOR) activity, lipogenic, protein and lactose gene expression, and milk composition of lactating sows. Forty lactating multiparous sows were randomly assigned to one of two treatments for 15 days (from day 13 to day 28 of lactation): 1) Control (no TZD added); 2) 8 mg/kg BW of TZD mixed with feed. TZD did not affect insulin, glucose and non-esterified fatty acids (NEFA) in the plasma. There was no effect on milk composition, however, TZD reduced saturated fatty acids (SFA; P = 0.02) and increased monounsaturated fatty acids (MUFA; P = 0.03) by 11.3 and 13.7% in the milk FA profile, respectively. In addition, there was an increase of 20.1% in the expression of AGPAT6 (P = 0.001) and trends of DGAT1 (P = 0.08) and SCD (P = 0.06) in the mammary gland of TZD-treated sows. Lipogenic genes in adipose tissue were not affected. Among the genes related to protein synthesis in mammary gland tissue, CSN3 increased by 42.7% (P = 0.02) in TZD compared to Control. In summary, TZD increases the expression of genes related to lipid and protein synthesis in the mammary gland and alters SFA and MUFA in the milk profile of lactating sows.

CHAPTER III

MODULATION OF THE PLASMA LIPIDOMIC PROFILE IN PIGLETS FED POLAR LIPID-RICH DIETS

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Article

Modulation of the Plasma Lipidomic Profile in Piglets Fed Polar Lipid-Rich Diets

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Abstract: Background: Polar lipids from dairy are novel sources of energy that may replace other dietary lipids and impact plasma lipidomic profiles in piglets. This study evaluated the impact of feeding diets rich in polar lipids on the plasma lipidome of piglets during the weaning period. **Material and Methods:** Weaned male piglets (n = 240; 21 days of age; 6.3 ± 0.5 kg of BW) were blocked by initial weight and distributed into 48 pens of five animals each in a complete randomized block design with a 2 × 3 factorial arrangement of treatments as follows: a plant-based diet rich in neutral lipids from soybeans (24 pens; SD) or a polar lipid-rich diet by-product of cheese making (24 pens; PD) from weaning until the 21st day of the nursery phase. Within each diet group, animals received one of three milk replacers (MR; 0.5 L/d/animal) for the first 7 days after weaning: (1) commercial MR containing animal and coconut lipids (CO); (2) polar lipid-based MR (PO); or (3) soybean lipids-based MR (SO). **Results:** The PD diet group increased the plasma concentrations of sphingolipids, phospholipids, and cholesterol esters, but did not impact the concentrations of glycerolipids (GLs). Both the PO and CO milk replacers increased the plasma concentrations of ceramide, acyl-chain phosphatidyl choline, and cholesterol esters. The plasma concentrations of GLs containing 18-carbon fatty acids such as 18:0, 18:1, 18:2, and 18:3, were higher in SD, whereas GLs containing 16:0 and 20:3 were higher in PD. **Conclusions:** In summary, the diet lipid type significantly modulated the plasma lipid composition in piglets 7 days after weaning. The dietary inclusion of polar lipids in diets for growing pigs can modulate the plasma lipidomic profile, relative to plant-based diets rich in soybean lipids. Cost may be a major consideration when using these lipids in pig diets. Their health benefits need to be further characterized in other models of stress and inflammation.

Keywords: plant-based feed; sphingolipids; phospholipids; piglet; plasma lipidome

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1. Introduction

The abrupt transition from a liquid to a solid diet during the weaning period leads to a reduction in feed intake and changes in the intestinal structure in piglets [1]. Villous atrophy and crypt elongation impair the function of the small intestine, increasing permeability and triggering inflammatory processes [2]. Potentiated by psychosocial and immunological stressors, inflammation compromises the health and performance of animals [3].

Diet, as a modulator of different aspects of intestinal health, can enhance or minimize these inflammatory effects on the basis of its composition [4]. Vegetable protein sources such as soy can be used in piglet diets, but the potential for digestive issues limits their inclusion. However, in addition to being challenging to the immature intestine of young

piglets, these dietary ingredients contain antinutritional factors and proinflammatory n-6 lipids, such as linoleic acid (LA; 18:2 *cis*-9, *cis*-12; [3]). Although soybeans contain lipids with anti-inflammatory functions (e.g., sphingolipids), their concentrations are typically low (1.6–2.0% phospholipids in whole beans; [5]) compared to polyunsaturated fatty acid (PUFA)-rich triglycerides (TGs), which represent around 64% of total lipids [6]. Furthermore, the high proportions of n-6 PUFA present in grains and vegetable oils can increase oxidative stress and the production of proinflammatory mediators, mainly through the arachidonic acid pathway [7]. Pigs fed a diet with high n-6/n-3 ratios showed lower energy digestibility and weight gain rates [8], which could have been associated to reduced inflammation and changes in metabolism [9].

On the other hand, polar lipids from the globular membranes of bovine milk fat, such as glycerophospholipids and sphingolipids, are lipid sources that can induce anti-inflammatory and protective effects on intestinal integrity [10]. Sphingomyelin is present in approximately 75% of bovine milk sphingolipids [11]. Young mice fed a lard-based diet containing 0.1% by weight of sphingomyelin of bovine dairy origin for 10 weeks had reduced hepatic accumulation of triglycerides and circulating inflammatory cytokines, attenuating the inflammatory responses of macrophages after pro-inflammatory stimuli [10]. Furthermore, the supplementation of polar lipids from milk fat globular membranes during gestation resulted in reduced gut permeability and the improved growth of neonatal pigs [12]. Sphingomyelin exhibits antimicrobial, antioxidant, and neuroprotective effects [13], and it can be a substantial dietary source of phosphocholine, and, thus, promote neonatal development [14]. Sphingosine-1-phosphate (S1P) is the phosphorylated product of sphingosine, the backbone of sphingomyelin and other sphingolipids. Importantly, S1P is considered a regulator of inflammation given its roles in reducing T-cell proliferation and cytokine synthesis in the intestinal tract [15].

Lipidomics is a powerful tool for investigating the modulation of metabolism during normal and disease states. Understanding the response of cellular metabolism to a dietary intervention by quantifying changes in diverse lipid classes and species can be associated to changes in gene expression and enzyme activity [16], which can improve our understanding of the potential impact of diet on performance and animal health. Since complex dietary lipids such as sphingolipids are not absorbed intact in the intestine [17], the plasma concentration of hydrolyzed lipids could serve as a proxy for their digestion and metabolism.

We hypothesized that the polar lipids in the diet and milk replacers would result in extensive changes in the plasma lipidomic profile of growing piglets, which can be associated with modulation of inflammatory markers during the first 7 days after weaning. The objective was to evaluate the impact of a modified by-product of cheese-making rich in polar lipids on the plasma lipidomic profiles of piglets during the weaning period. In addition, we aimed to study the influence of the dietary fatty acid profile on plasma lipid composition.

2. Materials and Methods

2.1. Experimental Design and Treatments

All procedures were approved by the animal care committee (2022-PO-440) of the Centre de Recherche en Sciences Animales de Deschambault (CRSAD), in Deschambault, Canada, following the regulations of the Canadian Council on Animal Care (1993).

A total of 240 weaned male piglets (25% Landrace, 25% Yorkshire and 50% Duroc) of 21 days of age were used in this study. Animals were blocked by initial weight (6.3 ± 0.5 kg) and distributed into 48 pens of five animals in a complete randomized block design with a 2 × 3 factorial treatment arrangement. From the start of the nursery phase, animals received one of two diets ad libitum: (1) soy diet (SD), a feed containing soy lipids where sphingomyelin (SM), phospholipids containing choline (PC), and triglyceride (TG) were 0.40, 18, and 44% of total lipids, respectively (24 pens); or (2) polar diet (PD), a diet

containing polar lipids from a phospholipid- and protein-rich by-product of cheese making (Iso-Chill 6000, Agropur, Dairy Cooperative. St-Hubert, QC, Canada), where SM, PC, and TG were 13, 27, and 44% of total lipids, respectively (24 pens). From the start of the nursery phase to day 7, animals within each dietary group were offered one of three milk replacers (0.5 L/pig/day; consumed completely in all groups): control milk substitute (CO), a commercial product composed of animal fat lipids and coconut oil (SM, PC, and TG were 11, 27, and 50% of total lipids, respectively); (2) milk substitute rich in polar lipids (PO), containing a high-fat whey protein concentrate (Isochill 6000, Agropur, QC, Canada; SM, PC, and TG were 21, 36, and 22% of total lipids, respectively); or (3) milk substitute composed of vegetable lipids (SO), containing lipids from soybeans (SM, PC, and TG were 0.2, 0.8, and 75% of total lipids, respectively). Lipid concentrations, including sphingolipids, glycerolipids, phospholipids, and sterol lipids, are shown Figure 1 and Supplemental Table S1. All piglets received vaccines for Porcine Circovirus type 2 (Ingelvac Circoflex; Boehringer Ingelheim Ltd., Burlington, ON, Canada) and *Mycoplasma Hyopneumoniae* (Ingelvac Mycoflex; Boehringer Ingelheim Ltd., Burlington, ON, Canada). A total mortality rate of 1.6% was observed during the experiment, corresponding to one animal in the CO group, one in the PO group, and two in the SO group.

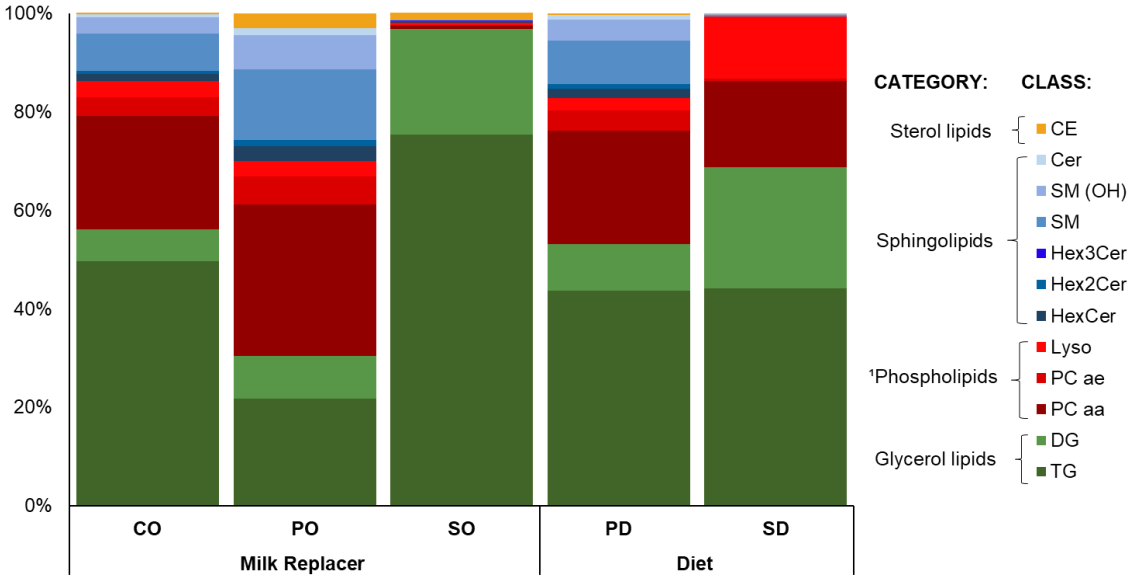

Figure 1. Overall concentration of diverse lipid classes identified in experimental milk replacers and diets. Treatments were (1) milk replacer, commercial milk substitute rich in animal fat and coconut oil (CO), milk substitute rich in polar lipids, (PO) or milk substitute rich in soy lipids (SO); and (2) diet, solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD). Sterol lipids: cholesteryl ester (CE). Sphingolipids: ceramide (Cer); sphingomyelin (SM); sphingomyelin with a hydroxyl group (SM (OH)); ceramide with a hexose sugar residue attached to the sphingoid base and a fatty acid chain (HexCer); ceramide with two hexose sugar residues attached to the sphingoid base and a fatty acid chain (Hex2Cer); and ceramide with three hexose sugar residues attached to the sphingoid base and a fatty acid chain (Hex3Cer). ¹Phospholipids containing choline: phosphatidylcholine with an acyl chain (PC aa); phosphatidylcholine with an acyl-alkyl chain (PC ae); and lysophosphatidylcholine (Lyso). Glycerolipids: diacylglyceride (DG) and triglyceride (TG). The details in lipid composition of treatments as % of total lipids are shown in Supplemental Table S1.

The feed was formulated based on NRC (2012) requirements for digestible amino acids and metabolizable energy. Diets were formulated to be iso-energetic and iso-proteic. Feed samples were collected once a week and stored at −20 °C until further analyzed.

Blood samples were collected on day 7 from three randomly selected piglets in every pen. Samples were kept on ice and centrifuged within 30 min at 4 °C 3000× *g* to obtain

plasma, which was then pooled by pen (n = 8 pooled samples per treatment) and conserved at −80 °C until analyzed for the concentrations of lipopolysaccharide-binding protein (LBP) by immunoassay (MyBiosource; Swine LBP ELISA kit) and for lipidomic analyses as described below. Fecal samples from three animals per pen were collected on day 7 after placing piglets in individual crates where they were allowed to defecate. These samples were pooled by pen, snap-frozen, and stored at −80 °C until analyzed for calprotectin concentrations by ELISA (MyBiosource; Swine Calprotectin kit).

2.2. Feed Composition Analyses

Diets and milk replacers were sampled once per week and pooled by treatment for the experimental period, then analyzed for humidity, crude protein, crude fiber, and minerals by wet chemistry (SGS Canada Inc., Guelph, ON, Canada; Table 1). Fatty acids (FA) were directly trans-esterified [18] and methyl esters were identified and quantified in a gas chromatograph (Agilent 7820A; Agilent Technologies Canada Inc. Mississauga, ON, Canada) equipped with a HP-INNOWax column (30-m length × 0.32-mm internal diameter × 0.25-µm film thickness; Agilent Technologies Canada Inc.) and a flame ionization detector with hydrogen as the carrier gas [19]. Total fatty acid content of feeds was determined using 13:0 and 21:0 as internal standards (Sigma Aldrich, Diegem, Belgium). Dietary fatty acid profiles are shown in Table 2.

Table 1. Ingredient and chemical compositions of experimental milk replacers (MR) and diets.

Ingredients, %	MR			Diet	
	CO	PO	SO	PD	SD
Lactoserum powder	35.9	51.2	46.6	-	-
Ground corn	-	-	-	21.9	28.9
WPC 50% [2]	-	31.5	-	20.0	-
Ground wheat	-	-	-	18.2	-
Wheat flour	-	-	-	-	14.6
Corn gluten meal	-	-	-	15.0	14.6
Ground beet pulp	-	-	-	10.0	-
Soy protein concentrate	-	-	19.1	-	11.6
Ground soybean meal 48% CP	-	-	-	-	9.75
Animal fat	23.8	9.5	11.0	-	-
Coconut fat	0.9	3.8	-	-	-
WPC 80% [3]	21.4	-	11.0	-	-
WPC 34% [4]	15.1	-	-	-	-
Soybean oil [5]	-	-	9.2	-	-
Dicalcium de phosphate	1.2	-	1.3	-	-
L-Lysine	-	1.0	-	-	-
Others	1.7	3.0	1.8	4.83	8.59
Chemical composition, %				-	-
Humidity	4.5	4.5	4.9	9.49	9.45
Crude Protein	28.0	27.6	26.6	19.4	21.2
Fatty acids	15.3	15.4	16.4	6.32	7.65
Crude fiber	<0.1	0.2	0.2	4.29	3.07
Metabolizable energy (Mcal/kg)	4.0	4.1	4.2	3.39	3.52
Ash	6.7	6.0	6.8	5.37	5.63
Calcium	0.5	0.6	0.6	0.71	0.61
Phosphorus	0.6	0.6	0.7	0.60	0.72
Potassium	1.0	1.2	1.4	0.40	0.84
Magnesium	0.03	0.04	0.1	0.12	0.13
Sodium	0.8	0.7	0.7	0.25	0.39

Treatments were (1) milk replacer, commercial milk substitute rich in animal fat and coconut oil (CO), milk substitute rich in polar lipids (PO), or milk substitute rich in soy lipids (SO); and (2) diet, solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD). [2] Whey protein concentrate (50% protein; Isochill 6000; Agropur Dairy Cooperative, St-Hubert, QC, Canada). [3] Whey protein concentrate (80% protein). [4] Whey protein concentrate (34% protein). [5] Contained 7.8% lysolecithin.

Table 2. Fatty acid profiles of experimental milk replacers (MR) and diets.

	MR			Diet	
	CO	**PO**	**SO**	**PD**	**SD**
FA % of DM	15.30	15.43	16.39	6.32	7.65
FA profile % of total FA					
8:0	1.56	2.04	1.41	0.61	0.08
10:0	1.46	2.46	1.34	1.52	0.11
12:0	9.93	12.14	8.79	1.94	0.36
14:0	5.75	9.55	5.31	6.59	0.44
14:1	0.10	0.38	0.11	0.54	0.06
15:0	0.16	0.59	0.18	0.83	0.08
16:0	21.79	25.22	20.99	26.53	13.59
16:1	1.74	1.49	1.60	1.15	0.23
17:0	0.27	0.37	0.27	0.43	0.12
18:0	10.60	9.98	9.97	8.13	3.41
18:1.*cis*-9	31.49	24.23	30.04	23.89	25.50
18:1.*cis*-11	2.21	1.79	2.10	1.31	1.20
18:2n6	10.24	6.90	14.40	22.51	48.62
18:3n6	0.09	0.10	0.08	0.13	0.05
18:3n3	0.43	0.39	1.05	0.99	4.60
20:0	0.19	0.18	0.21	0.27	0.38
20:4n6	0.17	0.22	0.16	0.25	0.03
22:00	0.05	0.07	0.09	0.18	0.30
22:5n3	0.07	0.07	0.08	0.08	0.03
22:6n3	0.04	0.06	0.04	0.09	0.01
Unidentified	1.65	1.78	1.79	2.02	0.79

Treatments were (1) milk replacer (MR), commercial milk substitute rich in animal fat and coconut oil (CO), milk substitute rich in polar lipids (PO), or milk substitute rich in soy lipids (SO); and (2) diet, solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD). FA, fatty acid; DM, dry matter.

2.3. Feed and Plasma Lipidomic Analysis

A lipidomic analysis was performed on feed and plasma samples for determination of cholesteryl ester (CE), ceramide (Cer), sphingomyelin (SM), sphingomyelin with a hydroxyl group (SM (OH)), ceramide with a hexose sugar residue attached to the sphingoid base and fatty acid chains (HexCer), ceramide with two hexose sugar residues attached to the sphingoid base and fatty acid chains (Hex2Cer), ceramide with three hexose sugar residues attached to the sphingoid base and fatty acid chains (Hex3Cer), phosphatidylcholine with an acyl chain (PC aa), phosphatidylcholine with an acyl-alkyl chain (PC ae), lysophosphatidylcholine (Lyso), diacylglyceride (DG), and triglyceride (TG) concentrations (The Metabolomics Innovation Center; AB, Canada). Feed samples were homogenized with three-fold volume of extraction buffer consisting of 85 mL of MeOH + 15 mL of phosphate buffer (10 mM). Samples were then centrifuged at 14,000 rpm and supernatants used in the lipidomics assay were as described for plasma below.

Plasma samples were thawed on ice in the dark before use. In a 96-well filter plate, 20 μL of the internal standard mixture solution [including 9:0 Lyso PC, 6:0 SM, 14:0 PC, Cer(d18:0/12:0(OH)), CE(16:0)-d7, DG(17:0_17:0)-d5, GlcCer(d18:1/12:0), and TG(16:0_34:0)-d5 as internal standards; and 18:0 Lyso PC, 18:0 SM, 36:0 PC, Cer(d18:1/18:0), CE(17:0), DG(18:1/18:1), LacCer(d18:1/18:0), GlcCer(d18:1/18:0), and TG(18:1/18:1/18:1)] and 10 μL of sample was pipetted directly onto the center of the spot. The plate was evaporated for 30 min to dryness under nitrogen, followed by the addition of 300 μL of methanol containing 5 mM ammonium acetate. The plate was covered and shaken at 450 rpm for 30 min at room temperature, and then centrifuged for 5 min at 500 rpm. After centrifugation, 10 μL of the extracts was transferred to a new 96-well collection plate and mixed thoroughly with 490 μL of running solvent (60 μL of formic acid and 10 mL of water in 290 mL of methanol). Then, 20 μL was injected into an Agilent 1290 series Ultra high-performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with a tandem mass spectrometry instrument (ABSciex

5500 QTrap®, Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) equipped with an Ultra high performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA, USA). Data analysis was performed using MultiQuant TM 3.0.3 (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA). The UHPLC autosampler was connected directly to the MS ion source by red PEEK tubing. The running buffer was used as the mobile phase, while the flow rate was programmed as follows: t = 0 to 1.6 min, 30 μL/min; t = 2.4 to 2.8 min; 200 μL/min; and t = 3.0 min, 30 μL/min. The sample injection volume was 20 μL. The IonSpray voltage was set at 5500 V and the temperature was set at 200 °C. The curtain gas, gas pressure 1, gas pressure 2, and collision-induced dissociation were set at 20, 40, 50, and medium, respectively. The entrance potential and collision cell exit potential were set at 10 and 15 V, respectively.

2.4. Statistical Analysis

Data were analyzed using the web-based platform MetaboAnalyst 5.0 [20]. Non-filtered data were normalized by the sum method, generalized log-transformed, and Pareto-scaled. Multivariate analysis of data included partial least squares discriminant analysis (PLS-DA), ANOVA, and Pearson's correlation coefficient procedures. Significance was declared at a false discovery rate (FDR) <0.05. For visualization purposes, heat maps were generated to showcase the magnitude of fold-change in a color gradient for increased (red) or decreased (blue) relative abundance.

Data found to be significantly affected by treatments were then analyzed in a mixed model in SAS 9.4 (The SAS Institute Inc., Cary, NC, USA) including pen as a random effect, and milk replacer, a diet, and their interactions were parametrized as fixed effects. Significant differences were considered when $p \leq 0.05$ for main effects and $p \leq 0.10$ for interactions; trends for differences were considered when $p \leq 0.10$ for main effects and $p \leq 0.15$ for interactions.

3. Results

The reversed-phase LC-MS/MS assay identified 404 lipids in the diet (Figure 1; Supplemental Tables S1 and S2) and in the plasma of piglets (Supplemental Table S3) that can be grouped into 12 classes belonging to four lipid types: sphingolipids, sterol lipids, a subgroup of phospholipids (i.e., those containing choline), and glycerolipids. The sphingolipids detected included 9 SM, 5 SM (OH), 18 Cer, 17 HexCer, 7 Hex2Cer, and 4 Hex3Cer. The phospholipids subgroup included 14 Lyso, 37 PC aa, and 38 PC ae. The glycerolipids included 21 DG and 212 TG. Finally, sterol lipids were represented by 22 CE.

In solid feeds, the lipid class concentrations varied according to the composition of each dietary treatment (Figure 1; Supplemental Table S1). Relative to SD, the PD diet contained higher concentrations of sphingolipids (i.e., 31-fold) and cholesterol esters (i.e., five-fold), equal concentrations of phospholipids, and lower concentrations of glycerolipids (i.e., −23%). Similarly, relative to CO and SO, the PD milk replacer contained higher concentrations of sphingolipids (i.e., two- and 40-fold, respectively), cholesterol esters (i.e., 11- and two-fold, respectively), and phospholipids (1.3- and 31-fold), and lower concentrations of glycerolipids (i.e., −46% and −69%).

The fatty acid (FA) profiles of the treatments showed higher concentrations of 12:0, 14:0, and 16:0 in polar lipid-rich treatments (PO and PD; Table 2). Meanwhile, CO and SO milk replacers and the SD diet presented a higher concentration of 18:1. In addition, the SD diet group exhibited higher concentrations of 18:2n6 and 18:3n3 compared with PD.

Effects of Milk Replacer and Diet on Plasma Lipids of Weaned Piglets

Two-factor analysis revealed no interaction between milk replacer and diet for plasma lipids (*FDR* > 0.05). The factors were then analyzed individually.

Partial least squares discriminant analysis (PLS-DA) plots of the plasma lipid data revealed no clear clustering by MR group (i.e., CO, PO and SO) (Figure 2A). However, the variable importance projection (VIP) scores analysis showed that CE, TG, Cer, Hex2Cer, and HexCer had VIP scores > 1 (Figure 2B). These lipids were highest in CO, medium in PO, and lowest in the SO milk replacer treatment. The heatmap revealed no clear clustering of lipid type concentrations by milk replacer (Figure 2C). The heatmap clustering (Figure 2D) shows higher concentrations of calprotectin and LBP in the SO group compared to other MR, although mixed-model analysis revealed no significant differences.

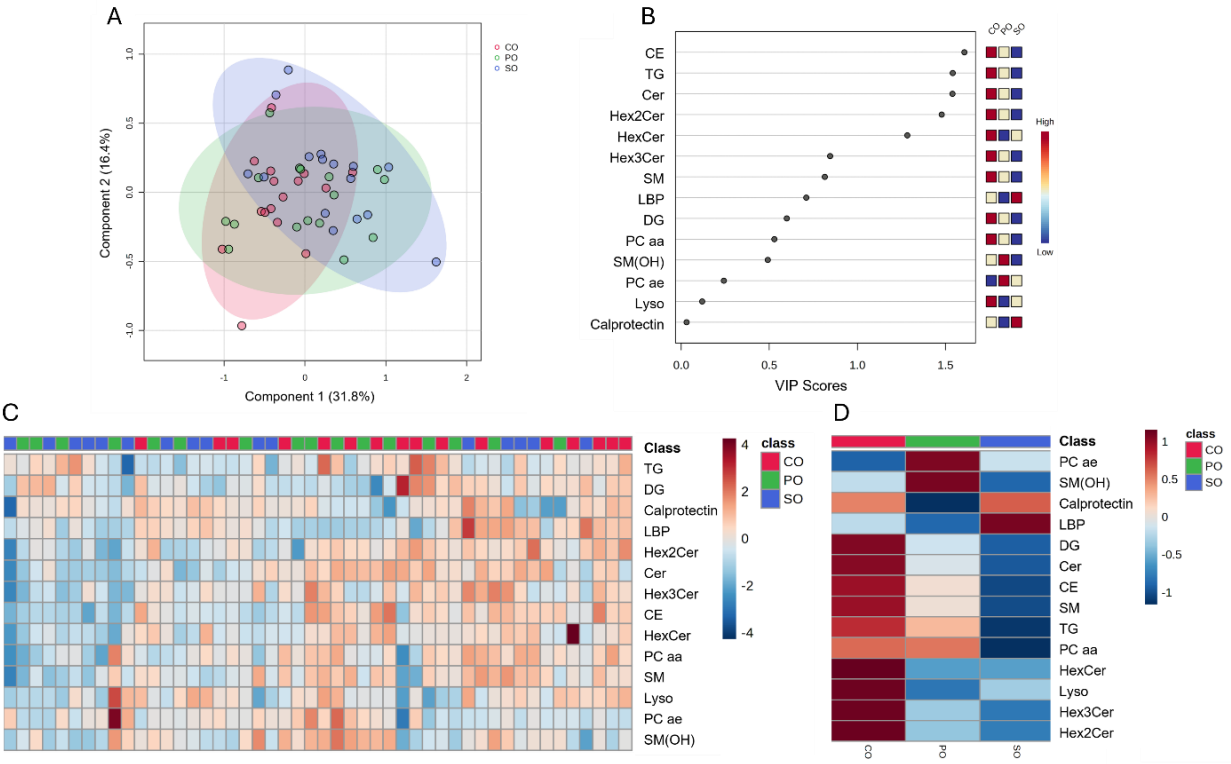

Figure 2. Discriminant analysis and hierarchical grouping of plasma lipid classes measured on day 7 of the nursery phase in piglets fed commercial milk substitute rich in animal fat lipids and coconut oil (CO), milk substitute rich in polar lipids (PO), or milk substitute rich in soy lipids (SO). (**A**): two-dimensional partial least squares discriminant (PLS-DA) score plot. (**B**): variable importance projection (VIP) scores analysis based on component 1 of the PLS-DA used to rank the relative contribution of lipids to the variance between treatments. (**C**): heatmap clustering analysis of the plasma lipid classes influenced by milk replacer treatments. (**D**): heatmap group averages. Plasma lipids data were obtained using a LC–MS/MS custom assay.

Similarly, the PLS-DA plots of the plasma lipid data revealed unique features predictive of SD and PD diets (Figure 3A). The VIP scores analysis showed that only Cer had a VIP score > 1 (Figure 3B), being the highest in the PD treatment. Heat maps showed no clear clustering of lipid type concentrations by diet (Figure 3C). The heatmap analysis (Figure 3D) also showed higher concentrations of calprotectin and LBP in the SD compared with the PD group. However, when the 404 lipids identified in piglets' plasma were analyzed individually, the discriminant analysis revealed a clear clustering by diet type (PD and SD; Figure 4A). The VIP scores analysis showed the top 15 lipids that had a VIP score > 1, namely, TG containing 16:0, 18:0, 18:1, 18:2, 18:3, and 20:3 fatty acids (Figure

3B). The heatmap analysis of the 25 top clustered lipids (Figure 4C,D) showed that TG containing fatty acids such as 16:0, 17:1, 18:0, 18:1, 18:2, and 18:3 were elevated in the SD group, as were DG containing 18:1/18:2 and 18:2/18:2 fatty acids. On the other hand, the PD group exhibited a higher concentration of TG containing 20:3 carbon fatty acids.

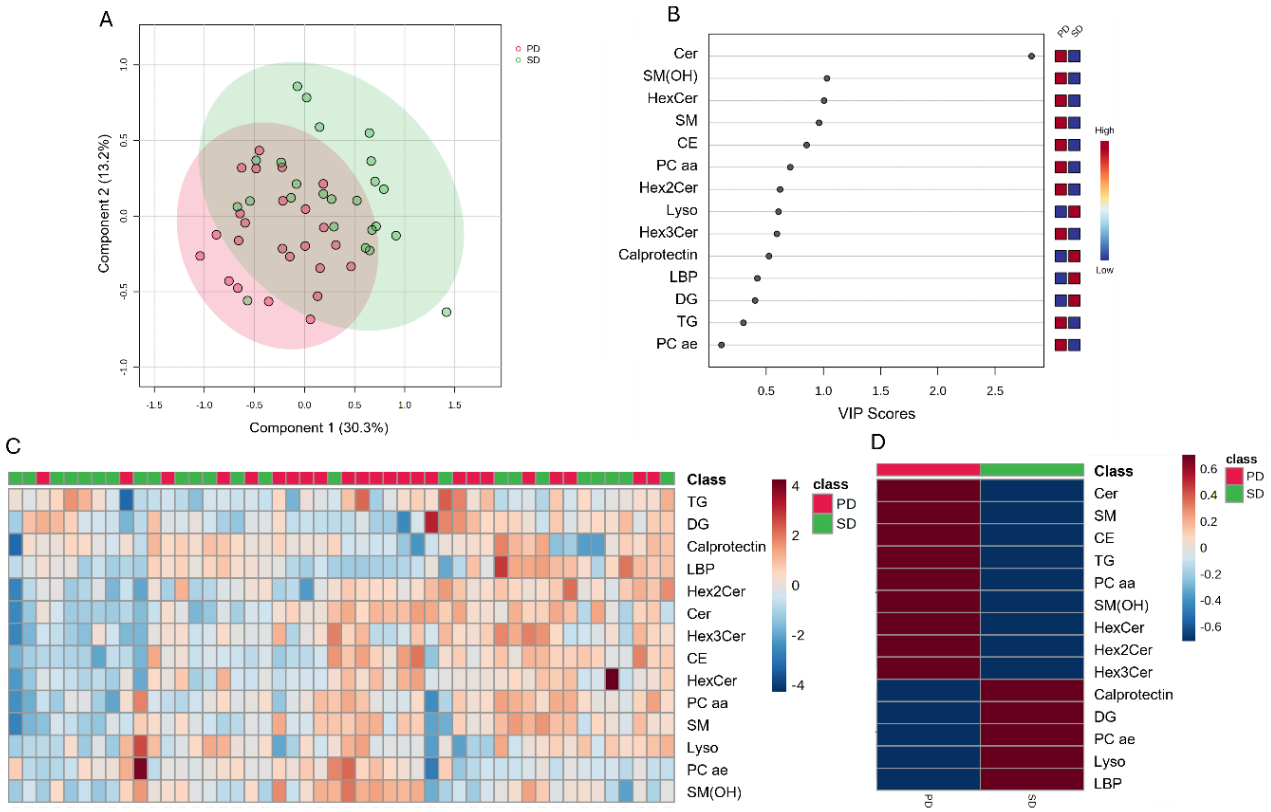

Figure 3. Discriminant analysis and hierarchical grouping of plasma lipid classes measured on day 7 of the nursery phase in piglets fed a diet rich in soy lipids (SD) or a diet rich in polar lipids from cow's milk fat globular membranes (PD). (**A**): two-dimensional partial least squares discriminant (PLS-DA) score plot. (**B**): variable importance projection (VIP) scores analysis based on component 1 of the PLS-DA used to rank the relative contribution of lipids to the variance between treatments. (**C**): heatmap clustering analysis of the plasma lipid classes influenced by treatments. (**D**): heatmap group averages. Plasma lipids data were obtained using a LC–MS/MS custom assay.

The mixed-effects model analysis revealed an effect of both diet and milk replacers on the plasma concentrations of CE, Cer, HexCer, Hex2Cer, Hex3Cer, PC aa, and SM (OH; $p < 0.05$; Table 3), whereas glycerolipids remained unchanged. More specifically, compared with the SD diet group, PD resulted in higher plasma concentrations of SL, PC aa, and CE ($p < 0.05$). In addition, relative to SO, both the CO and PO milk replacer groups exhibited higher plasma concentrations of Cer, PC aa, and CE ($p < 0.05$), whereas SM(OH) concentrations were higher in PO compared to CO and SO ($p < 0.05$).

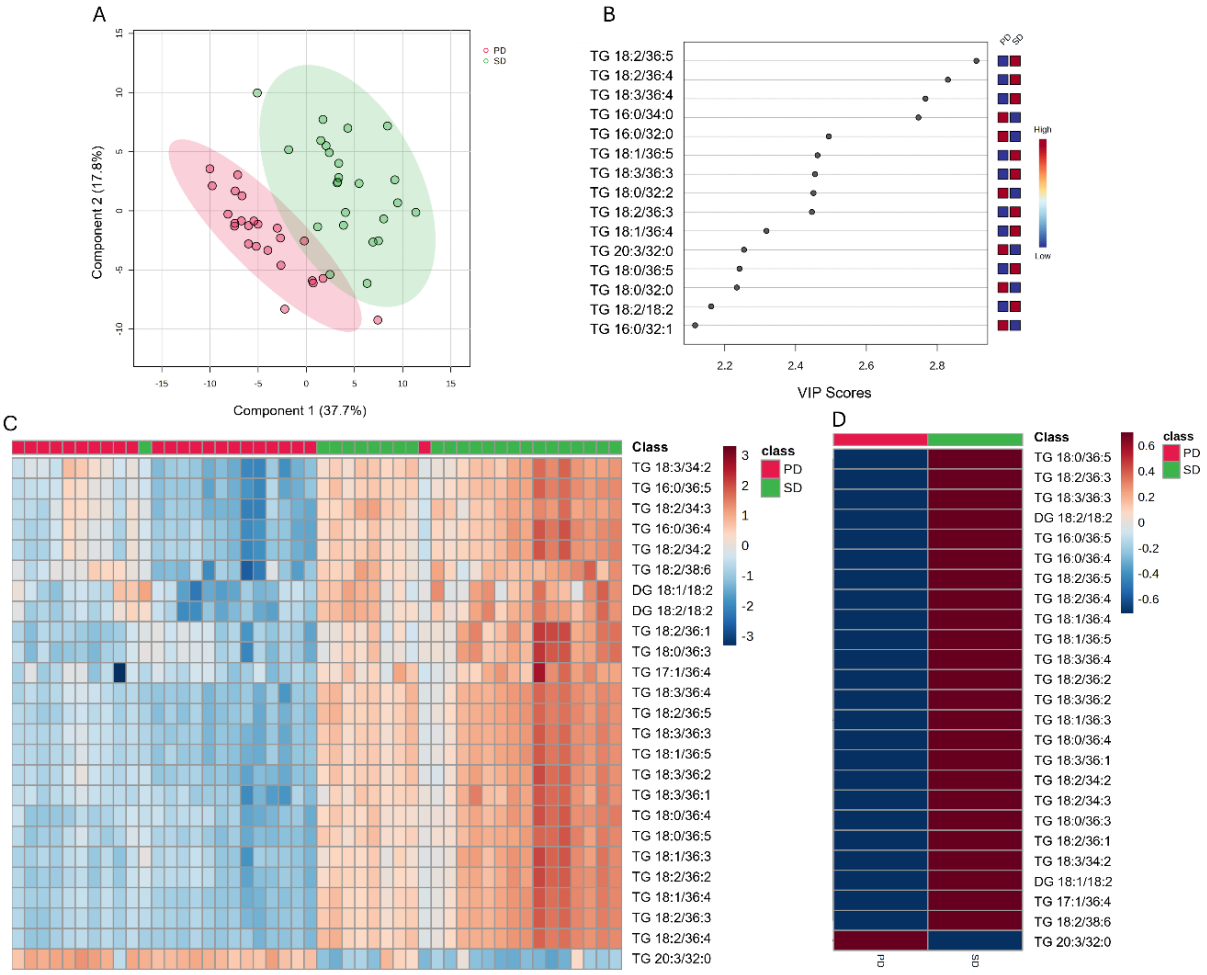

Figure 4. Discriminant analysis and hierarchical grouping of plasma lipid concentrations on day 7 of the nursery phase in piglets fed a diet rich in soy lipids (SD) or a diet rich in polar lipids from cow milk fat globular membranes (PD). (A): two-dimensional partial least squares discriminant (PLS-DA) score plot. (B): variable importance projection (VIP) scores analysis based on component 1 of the PLS-DA used to rank the relative contribution of lipids to the variance between treatments. (C): heatmap clustering analysis of the plasma lipid classes influenced by milk replacer treatments. (D): heatmap group averages. Plasma lipids data were obtained using a LC–MS/MS custom assay.

Table 3. Effects of milk replacers (MR) and diets on the concentration of plasma lipid classes in piglets on day 7 of the nursery phase.

Lipid Category	Lipid Class	Milk Replacer			Diet			*p*-Value		
		CO	PO	SO	PD	SD	SEM	MR	Diet	MR x Diet
Sphingolipids	Cer µM	2.43 ^{a}	2.20 ^{a}	1.87 ^{b}	2.60 ^{A}	1.78 ^{B}	0.12	0.01	<0.0001	0.64
	HexCer µM	2.95 ^{a}	2.56 ^{ab}	2.38 ^{b}	2.84 ^{A}	2.42 ^{B}	0.14	0.04	0.02	0.42
	Hex2Cer µM	0.94 ^{a}	0.85 ^{ab}	0.76 ^{b}	0.89 ^{A}	0.80 ^{B}	0.03	0.002	0.02	0.94
	Hex3Cer µM	0.32 ^{a}	0.31 ^{ab}	0.28 ^{b}	0.32 ^{A}	0.29 ^{B}	0.01	0.02	0.01	0.23
	SM(OH) µM	9.83 ^{b}	10.92 ^{a}	8.84 ^{b}	10.55 ^{A}	9.16 ^{B}	0.47	0.01	0.02	0.56
	SM µM	133	129	114	135 ^{A}	116 ^{B}	6.33	0.09	0.01	0.64
Glycerolipids	DG µM	15.0	14.6	13.2	14.4	14.2	0.75	0.22	0.83	0.96
	TG µM	403	381	295	374	340	41	0.13	0.47	0.22
Phospholipids [1]	PC aa µM	635 ^{a}	644 ^{a}	572 ^{b}	650 ^{A}	584 ^{B}	20.8	0.03	0.01	0.83
	PC ae µM	67.0	71.3	64.4	69.1	66.0	2.87	0.27	0.37	0.60
	Lyso µM	84.1	84.3	78.7	82.6	82.0	3.32	0.39	0.88	0.66
Sterol lipids	CE mM	4.13 ^{a}	3.86 ^{a}	3.23 ^{b}	3.99 ^{A}	3.47 ^{B}	0.19	0.004	0.02	0.45

Treatments were (1) milk replacer, commercial milk substitute rich in animal fat and coconut oil (CO), milk substitute rich in polar lipids (PO), or milk substitute rich in soy lipids (SO); and (2) diet, solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD). Sphingolipids: ceramide (Cer); sphingomyelin (SM); sphingomyelin with a hydroxyl group (SM

(OH)); ceramide with a hexose sugar residue attached to the sphingoid base and a fatty acid chain (HexCer); ceramide with two hexose sugar residues attached to the sphingoid base and a fatty acid chain (Hex2Cer); and ceramide with three hexose sugar residues attached to the sphingoid base and a fatty acid chain (Hex3Cer). Glycerol lipids: diacylglyceride (DG); triglyceride (TG). [1] Phospholipids containing choline: phosphatidylcholine with an acyl chain (PC aa); phosphatidylcholine with an acyl-alkyl chain (PC ae); and lysophosphatidylcholine (Lyso). Sterol lipids: Cholesteryl ester (CE). Values followed by different uppercase letters indicate significant differences between dietary treatments, while different lowercase letters indicate significant differences between MR treatments ($p < 0.05$).

4. Discussion

The use of milk replacers during the weaning period is not a common practice in pig farming due to the increase in production costs. However, the use of milk replacer can reduce the oxidative stress associated with feeding adaptation and intestinal maturation that piglets face during weaning in intensive farming systems [10]. Our study investigated the impact of different types of milk replacers in the first seven days after weaning, as this is the most critical period of this adaptive phase. Indeed, given the drastic change in diet (i.e., liquid to solid), feed intake and growth are depressed, and animals can develop leaky gut, inflammation, and oxidative stress [21]. Although it is not a common practice to provide milk replacers to all animals during the nursery phase (i.e., after weaning), our interest was to offer a liquid source of dietary lipids to counter the low solid feed intake observed during the first week, and, thus, to maximize the ingestion of these lipids during this period.

We tested a source of polar lipids from milk fat globular membranes obtained as a by-product of cheese-making and a soy source rich in PUFA, since different dietary lipid precursors, when hydrolyzed and absorbed by the intestine, can influence tissue metabolism and inflammatory biomarkers in different ways [22]. Furthermore, an important aspect of considering the use of polar lipids as a potential feed ingredient in diet for piglets was the potential to be easily obtained from dairy-processing factories as a by-product of cheese making, which may make it a viable alternative to other sources of lipids and proteins depending on the market price. Importantly, the future use of these products in swine feeding will also require an evaluation of optimal inclusion levels and effects on performance.

We measured the plasma concentrations of diverse lipid classes on day 7 of the experiment as the cumulative effect of diet and milk replacer was expected to be highest at this time. Our lipidomic analysis allowed to identify and quantify 404 different lipid types in the MR and diet treatments, which were grouped into the 12 classes. Globally, the predominant lipid type across treatments was glycerolipids (TG and DG), followed by sphingolipids (e.g., SM) and phospholipids (PC aa); however, the proportions varied greatly by treatment. Relative to milk fat globule membrane (MFGM) lipids, whole milk fat contains significantly higher concentrations of TG (i.e., 98% of total lipids), whereas the remainder is composed mainly of polar lipids, which, themselves, can vary significantly in composition [11,23]. Brink et al. [24] evaluated the variability in the composition of commercially available MFGM products for infants and found 338 lipid species. In their study, among the lipid classes with the highest relative abundance in a product derived from whey, TG was the highest, around 70%, followed by PC with 20% and SM 10%. In contrast, the PD diet contained 22, 31, and 14% of TG, PC, and SM, respectively, while the PO milk replacer contained 44, 23, and 9% of TG, PC, and SM, respectively. Such variation between available products may be the result of different manufacturing practices and substrates, which may be an important consideration since it may influence the impact of these products on animal metabolic and growth responses.

Our results did not demonstrate an interaction between the MR and diet treatments, but there were significant changes in plasma lipids when analyzed as individual factors. The descriptive analysis of the lipids grouped into 12 classes for MR and diet did not show

clear clustering; however, when the 404 lipids identified in the piglet plasma were analyzed individually, a clear clustering (i.e., metabolite grouping) by basal diet type was revealed, which indicates that dietary modifications are able to alter individual lipid species, perhaps reflecting changes in metabolism. Furthermore, the reason for treatment clustering only based on diet and not on the milk replacer type is not clear. Based on the amount of MR offered (0.5 L/animal/d), approximately 80 g of lipids from the MR were ingested. Although solid feed intake was not measured on day 7, typical intakes vary between 100 and 200 g/d, which would provide 6–12 g/d of lipids from the feed during this period. Therefore, diet clustering may be explained by other factors.

Among the 25 lipids with the highest plasma concentrations in SD, 23 were TG, mainly from species 18:0, 18:1, 18:2, and 18:3, and two were DG 18:1 and 18:2. Furthermore, the plasma concentration of glycerolipids (TG and DG) represented 68.7% of the total lipids identified in the SD treatment group. These results suggest a dietary origin, that is, a soy-based diet that predominantly has 18-carbon lipids in its composition, varying around 70% of the total FA depending on the soy source used [6]. Corroborating this, the dietary fatty acid profile demonstrated two and four times more 18:2n6 and 18:3n3, respectively, in the SD compared to the PD treatment. Combined with a lower concentration of 18:0 in the fatty acid profile of the SD group, it is suggested that the concentrations of 18:3, 18:2, and 18:1 present in plasma were of dietary origin, which presented a high plasma concentration in the SD plasma.

When comparing the FA profiles of the treatments, FA 18:0 presented a concentration twice as high in the PD diet profile compared to SD, and 18:1 presented similar concentrations in all treatments. Sphingosine with an 18-carbon chain and a cis double bond (d18:1) is the most abundant sphingoid base in mammalian cells [17], which explains the high concentrations of 18:1 in the fatty acid profile of diets of animal origin, PD, PO, and CO. Although 18:1 was equal in concentration to plant-based treatments, the fatty acid with the highest proportion in the lipid profile of polar lipids treatments (PD and PO) was 16:0. In addition, dietary 12:0 and 14:0 concentrations were higher in PO and PD, probably due the presence of coconut oil in these treatments [25]. Although medium-chain fatty acids are absorbed intact from the intestine [26], the top group-discriminant plasma lipids (TG and DG) did not contain these fatty acids.

The concentration of 16:0 was 1.9 times higher in PD, relative to the SD diet group, which was also reflected in a high transfer of this fatty acid to the plasma of piglets in PD (Figure 4B). Sphingolipids such as SM and Cer are not absorbed intact from the intestine; for example, SM is hydrolyzed by Alk-SMase to Cer and to sphingosine and free fatty acids (FA) by N-CDase in the intestinal lumen (Figure 5) [10]. Sphingosine, the backbone of most sphingomyelins, is absorbed well by the enterocyte. It is primarily phosphorylated to generate sphingosine-1-phosphate by sphingosine kinase (SoK; [27]). The remaining sphingosine is transported by chylomicrons to the lymph and blood. Sphingosine-1-phosphate can be dephosphorylated by S1P phosphatase (S1PP) to a new sphingosine to form Cer or degraded by S1P lyase to ethanolamine phosphate and hexadecenal [28]. Hexadecenal, in turn, is converted into free FA and can be, then, esterified into TG or CE (Figure 5). Furthermore, 16:0 can be activated by conversion to palmitoyl-CoA, and in turn, used to promote de novo ceramide synthesis by its condensation with serine residues [29]. While several molecular species may be synthesized, only a handful of them have been linked to disorders of metabolism. For example, C16:0-Cer is a pro-inflammatory molecule and it appears to play a role in the pathogenesis of obesity and diabetes [30]. Indeed, the fatty acid composition of different TG species will be dependent on diet lipid composition and will be associated to its potential to exert pro- or anti-inflammatory effects [31]. The identity of other FA in 16:0-containing TG is, therefore, expected to influence its overall effect on metabolism.

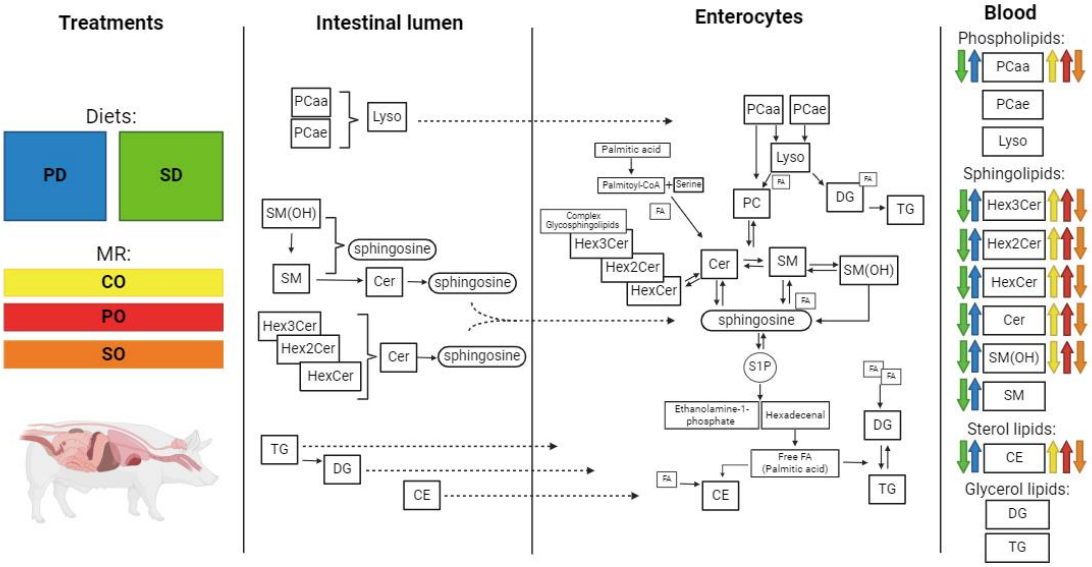

Figure 5. Digestion and absorption pathways of lipid classes identified in the diet and in milk replacer (MR) treatments. The treatments were (1) milk replacers, commercial milk substitute rich in animal fat lipids and coconut oil (CO), milk substitute rich in polar lipids (PO), or milk substitute rich in soy lipids (SO); and (2) diets, solid feed containing soy lipids (SD) or polar lipids from cow's milk fat globular membranes (PD). The intestinal lumen presents the classes of lipids identified from the analysis of dietary treatments and their possible metabolization before being absorbed into the enterocytes. Phosphatidylcholine with an acyl chain (PCaa) and phosphatidylcholine with an acyl-alkyl chain (PCae) are precursors of lysophosphatidylconine (Lyso) and can be transformed into Lyso in the intestinal lumen. Sphingomyelin with a hydroxyl group (SM(OH)) can be transformed into sphingomyelin (SM) or directly into sphingosine. SM is not absorbed into the intact enterocyte and is broken down into ceramide (Cer) in the intestinal lumen. Similarly, complex lipids such as ceramide with a hexose sugar residue attached to the sphingoid base and fatty acid chains (HexCer), ceramide with two hexose sugar residues attached to the sphingoid base and fatty acid chains (Hex2Cer), and ceramide with three hexose sugar residues attached to the sphingoid base and fatty acid chains (Hex3Cer) are not absorbed intact and are broken down into Cer. Cer is also hydrolyzed to sphingosine and fatty acids (FA). Sphingosine is then absorbed by enterocytes. Dietary triglycerides (TG) can be absorbed intact or broken down into diacylglycerides (DG) and free FA. Cholesterol ester (CE) is absorbed intact by the enterocyte. In the enterocyte, PCaa can produce Lyso or directly phosphatidylcholine (PC), which, together with Cer, constitutes SM. SM can acquire a hydroxyl group forming SM(OH) and be transformed back into Cer and PC, or it can be hydrolyzed to sphingosine. Cer can be transformed into SM when added to a PC, be hydrolyzed into sphingosine, or form complex sphingolipids via the salvage pathway. Sphingosine can be absorbed from the intestinal lumen or be the product of de novo synthesis from palmitic acid and serine. A small amount of sphingosine goes into the blood circulation and tissues to be integrated into SM and Cer or is catalyzed to generate sphingosine-1-phosphate (S1P). S1P is degraded to ethanolamine and hexadecenal phosphate. Hexadecenal is converted into palmitic acid, which can be esterified into DG and TG or transformed in CE. Blood has all identified lipid classes and the colored arrows represent an increase or decrease in their concentrations in the respective dietary treatments; green represents SD; blue represents PD; and yellow, red, and orange represent the CO, PO, and SO milk replacers. Lipid classes without colored arrows did not differ between treatments. Figure created in BioRender.com.

TG concentrations containing 20:3 were higher in the plasma of PD piglets. However, the FA profile of dietary feeds did not identify 20-carbon fatty acids with three unsaturations, which may be due to coelution with other long-chain fatty acids or metabolism into other FA. A small fraction of 20:3 can be desaturated to 20:4, while the main amount is normally metabolized via the cyclooxygenase pathway into series 1 prostaglandins, having anti-inflammatory actions [32].

Although it was the class of sphingolipids with the highest plasma concentration, SM was the only one that showed an effect from diet only, suggesting alterations in lipid

metabolism in MR treatments. As mentioned, dietary SM is not absorbed intact, that is, the amount found in plasma is endogenously reconstituted SM. SM is formed by Cer and a phosphocholine (PC) originating from the hydrolysis of lysophosphatidylcholine (Lyso) whose precursors are phosphatidylcholines (PCaa and PC ae; Figure 5). CE, phospholipids (PL), and TG represent 36, 30, and 16% of total lipids in plasma, respectively [33]. In swine plasma, phosphatidylcholine is the PL with the highest concentration, followed by SM and Lyso [29], which corroborates our findings (Table 2). Furthermore, PCaa was shown to be the main source of PC in the production of SM. Even though PCaa had the highest concentration among dietary phospholipids (Figure 1), this was the only lipid class in the phosphocholine category that was increased by the PD diet and also by the CO and PO milk replacers in MR.

Sphingomyelin, in addition to being reverted to Cer, can acquire a hydroxyl group (SM(OH))—which is also a direct source of sphingosine—and/or be hydrolyzed into sphingosine and free fatty acids (Figure 5) [17]. Hydroxylated SM, although less concentrated in plasma than SM, was regulated by diet and MR, being greater in the treatments containing polar lipids (PD and PO). Since SM was unaffected, but SM(OH), Cer, and PCaa were altered in the same treatment groups, this suggests that SM was metabolized through these pathways.

Plasma Cer, although found in lower concentrations compared with SM, was modulated by both diet and the MR treatments, being higher in the treatments with polar lipids (PD and PO) and CO. This class can be hydrolyzed by SoK to sphingosine and free FA, as previously described, but can also be a precursor of SM and complex lipids [17]. To the formation of complex sphingolipids through the salvage pathway, sugars are gradually added to their chain, forming HexCer, Hex2Cer, and Hex3Cer, respectively (Figure 5). This corroborates the gradual concentrations and differences between treatments for these classes, showing a Cer conversion.

Although not measured, an increase in S1P concentration is suggested due to the plasma increase in its precursors (i.e., SM present in animals fed polar lipid-rich feeds). S1P is a bioactive molecule with immunosuppressive and anti-inflammatory properties. It was expected that with the supply of SM in the diet, sphingosine phosphorylations would increase in the inflamed enterocytes of weaned piglets and reduce the proliferation of T cells and the synthesis of cytokines, controlling the inflammatory process and all its negative effects on metabolism [15]. Although our data on inflammatory markers did not show significant differences between treatments, it is likely that no underlying inflammatory processes were at play. Indeed, in contrast to commonly used models of weaning stress [21, 34, 35], ours included access to MR in all groups during the first week; animals had the opportunity to progressively adapt to the solid feed without relying exclusively on it from the start of the nursery phase. Although the plant-based lipids such as linoleic acid can act as precursors of pro-inflammatory oxylipids [36], the fact that the piglets ingested a milk replacer may have been enough to reduce the degrees of stress and inflammation typically observed during weaning.

5. Conclusions

Our results demonstrate large individual variation in plasma lipids according to dietary lipid composition in piglets 7 days after weaning. The lipid profiles of dietary treatments, in particular from the solid portion of the diet, altered the plasma lipid composition, resulting in increased circulating sphingolipids, phospholipids (i.e., PCaa), and cholesterol esters in the groups receiving polar lipid supplementation. Interestingly, changes in individual lipids were predominantly observed for TG and DG species, which corresponded with the dietary fatty acid profiles.

The inclusion of polar lipids in diets for growing pigs can modulate the plasma lipidomic profile relative to plant-based diets rich in soybean lipids. Future research is necessary to evaluate the extent of the impact of such changes in the plasma lipidomic

profile on overall metabolism, inflammation, and growth performance. In addition, the future use of polar lipids in the swine industry will depend on their availability as feeds and their market price in comparison to other sources of fat and protein.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Lipid composition of milk replacer and diet treatments as percentage of total lipids; Table S2: Concentrations (μM/g) of diverse lipid types identified in experimental milk replacers and diets; Table S3: Lipids identified in the plasma of piglets (μM) and their grouping into classes for lipidome analysis.

Author Contributions: The study was conceptualized by D.E.R. and J.E.R. Methodology and design were devised by D.E.R., J.E.R., M.-P.L.-M., J.L., and D.E.O., R.L. and S.C. applied all research protocols at the research farm. Data validation and analyses as well as writing of the original manuscript were performed by R.L., J.E.R., and D.E.R. All authors have read and agreed to the published version of the manuscript.

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CHAPTER IV

DIETARY POLAR LIPIDS ENHANCE GUT MICROBIOTA COMPOSITION AND ANTI-INFLAMMATORY LIPID MEDIATOR PROFILES IN WEANED PIGLETS

DIETARY POLAR LIPIDS IMPROVE GUT MICROBIOTA COMPOSITION AND ANTI-INFLAMMATORY LIPID MEDIATOR PROFILES IN WEANED PIGLETS

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ABSTRACT

Dairy polar lipids have demonstrated anti-inflammatory properties and protective effects on intestinal integrity, potentially mitigating the adverse impacts of weaning in piglets by modulating microbiota composition and intermediary metabolism. This study evaluated a dairy by-product rich in polar lipids on the microbiome and plasma lipid mediators of weaned piglets. A total of 240 male piglets (21 days old; 6.3 ± 0.5 kg) were assigned to 48 pens (5 animals/pen) in a randomized complete block design with a 2 × 3 factorial arrangement. Piglets received either a soybean lipid-based diet (SD; 24 pens) or a polar lipid-based diet (PD; 24 pens) from weaning to day 21. Within each diet, animals were provided one of three milk replacers (MR; 0.5 L/d)) for the first 7 days: 1) Commercial MR (CO; control); 2) Polar lipid-based MR (PO; polar); 3) Soybean lipid-based MR (SO). Animals were switched to a common diet from d 21 to 42. Plasma and fecal samples were collected for lipidomic and microbiome analyses. The PD diet significantly altered microbiota composition, increasing specific *Firmicutes*-associated genera (*Coprococcus, Roseburia*) while also modulating plasma lipid mediators. PD-fed piglets had higher levels of endocannabinoids (AEA, 2-AG), whereas the SD diet increased pro-inflammatory lipid mediators (13-HODE, 13-KODE) derived from linoleic acid. In conclusion, polar lipid supplementation in diet, but not in milk replacers, influenced

microbiota diversity and lipid mediator profiles, suggesting a potential long-term impact on immune regulation and metabolism. Future studies should explore these effects under more stressful weaning conditions.
Keywords: Sphingolipids; inflammation; microbiome composition; endocannabinoid system.

1 INTRODUCTION

Stress related to dietary, social and environmental changes during the weaning period causes intestinal disorders, dysbiosis, increases permeability and compromises gut functionality (MOESER; POHL; RAJPUT, 2017). Changes in the intestinal microbiota affect immune system function, modulating both pro-inflammatory and anti-inflammatory responses (PATIL; GOONERATNE; JU, 2020). These factors, combined with the metabolic changes that the animal undergoes at this stage, can compromise growth and performance throughout the piglet's life.

Gut microbiota also play a significant role in modulation of the endocannabinoid system, a network of lipid intermediates and receptors, capable of altering appetite, metabolism, and inflammation (MUCCIOLI et al., 2010). The arachidonic acid-derived endocannabinoids, 2-arachidonylglycerol (2-AG) and anandamide (*N*-arachidonoylethanolamine; AEA), interact with CB_1 and CB_2 cannabinoid receptors present on immune system cells and tissues to reduce the release of pro-inflammatory cytokines and modulate the immune response (KARWAD et al., 2017; RAKOTOARIVELO et al., 2024). In addition, the endocannabinoid system is involved in several physiological processes, including inflammation regulating energy balance, promoting metabolic processes, food intake, weight gain, fat accumulation in adipocytes and regulating body homeostasis (SILVESTRI; DI MARZO, 2013; TURCOTTE et al., 2015); thus, is also related to animal performance. Given the promiscuity of the enzymes that regulated the formation of 2-AG and AEA, other fatty acids can similar be utilized in the formation of related molecules that often signal through receptors other than the cannabinoid CB_1 and CB_2 receptors, and often have metabolic effects opposite to 2-AG and AEA;

this enlarged family of bioactive lipids (including 2-AG and AEA), their receptors and regulatory enzymes is termed the endocannabinoidome (eCBome) (DI MARZO, 2018; DI MARZO; SILVESTRI, 2019; VEILLEUX; DI MARZO; SILVESTRI, 2019). The eCBome is highly responsive to diets, especially dietary fatty acids, which can rapidly shift the synthesis of individual eCBome lipids and other lipids involved in cell including several types of oxylipins derived from omega-3 and -6 fatty acids (BOURDEAU-JULIEN et al., 2023; LACROIX et al., 2019).

Oxylipins are formed by enzymatic or non-enzymatic oxidation of polyunsaturated fatty acids (PUFAs) for which four main production pathways prevail: lipoxygenases (LOXs), cyclooxygenases (COXs), cytochrome P450 (CYPs), and reactive oxygen species (ROS; LIANG et al., 2024). The concentration and formation pathway of oxylipins correspond to the changes observed in their PUFA precursors. Furthermore, endocannabinoids and structurally related eCBome lipids can similarly be metabolized by these enzymes to their corresponding oxylipins (SIMARD et al., 2022). When sourced from omega-3 fatty acids, there is an increase in the production of anti-inflammatory oxylipins, while omega-6 fatty acids tend to promote the synthesis of pro-inflammatory oxylipins (CORAS et al., 2021; SHEARER; WALKER, 2018). Furthermore, the gut microbiome plays a crucial role in oxylipin synthesis. Specific microbial populations are known to similarly metabolize dietary fatty acids and are likely to influence the types and amounts of oxylipins produced (ÁVILA-ROMÁN et al., 2021; BECCACCIOLI et al., 2022; NIU; KELLER, 2019). This interaction suggests that diet and microbiome composition may together modulate oxylipin profiles, impacting inflammatory pathways and overall health (XU et al., 2022; PARCHEM et al., 2024).

Diet can modulate several aspects of gut health, including microbiota, composition, and intestinal permeability (BLAVI et al., 2021) and may be a useful strategy to mitigate weaning stress in piglets (WEI et al., 2021). Weaned piglets commonly have an abrupt switch from a digestible liquid milk to a solid corn-soybean-based diet. However, the digestive tract of weaned piglets is inefficient in metabolizing plant foods, and until the gut and microbiota composition are remodeled, feed efficiency and nutrient digestibility are low (WEI et al., 2021). On

the other hand, the addition of milk fat globule membrane and bioactive components to formula for neonatal piglets showed modulation of the microbiota in the colon and feces, reduced the proportion of opportunistic pathogens and increased the final body weight of piglets by 8% compared to a commercial formula (BERDING et al., 2016).

The milk fat membrane contains polar lipids, such as glycerophospholipids and sphingolipids (SL), composed of sphingomyelin. This molecule is related to anti-inflammatory (NORRIS et al., 2017), antioxidant and neuroprotective effects (KIM; AKBAR; KIM, 2010) and may indirectly promote neonatal development (ZEISEL; CHAR; SHEARD, 1986). However, little is known about how much the effect of sphingomyelin in modulating the microbiota can influence the immune system and the eCBome; whether these impacts differ from dietary compositions commonly used in pig farming practice. Furthermore, the gut microbiome and eCBome interact, with one altering the composition of the other, which are relevant to adiposity levels and glucose regulation (DIONE et al., 2020; MANCA et al., 2020; SURIANO et al., 2023).

We hypothesized that, first, lipids from a diet composed of sphingolipids would have a different impact on the composition of the intestinal microbiota compared to a soy-based diet and, second, that changes in the composition of the microbiota would be associated with the modulation of several types of lipids, including eCBome lipids and those related to inflammation and animal performance. The objective was to evaluate the use of a dairy processing byproduct rich in polar lipids in the microbiome and plasma lipid mediators of piglets during the weaning period.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL DESIGN AND TREATMENTS

All procedures were approved by the animal care committee (2022-PO-440) of the Centre de Recherche en Sciences Animales de Deschambault (CRSAD), following the regulations of the Canadian Council on Animal Care (1993).

Two hundred and forty weaned male piglets (25% Landrace, 25% Yorkshire and 50% Duroc) on 21 days of age, were blocked by initial weight (6.3 ± 0.5 kg) and distributed into 48 pens of 5 animals in a complete randomized block design with a 2x3 factorial arrangement. From day 0 to day 21, the animals received: 1) Soy diet (SD): feed containing soy lipids where sphingomyelin (SM), phosphatidylcholine (PC), and triglyceride (TG) were 0.40, 18, and 44% of total lipids, respectively (24 pens) or 2) Polar diet (PD): diet containing lipids from cow's milk fat globules where SM, PC, and TG were 13, 27, and 44% of total lipids, respectively (24 pens). Within each diet group from day 0 to 7, animals received 1 of 3 milk replacers: Control milk substitute (CO): commercial product composed of animal fat lipids and coconut oil where SM, PC, and TG were 11, 27, and 50% of total lipids, respectively (16 pens); 2) Milk substitute rich in polar lipids (PO): product composed of 25% of polar lipids derived from cow's milk fat globules (ISO Chill 6000, Agropur) where SM, PC, and TG were 21, 36, and 22% of total lipids, respectively (16 pens); or 3) Milk substitute composed of vegetable lipids (SO): soy-based product where SM, PC, and TG were 0.2, 0.8, and 75% of total lipids, respectively (16 pens). A 2-phases program was offered. In the first phase, diet treatments were applied from day 0 to 21. While in the second feeding phase, from day 21 to 42, all piglets received a common commercial pelleted feed. In both 2-phases, all feed and water were provided *ad-libitum*.

The feed was formulated based on digestible amino acids and metabolizable energy. Equal levels of energy and protein were provided. Diet composition, fatty acid profile and the complete lipid classes of treatments are shown in Larsen et al. (2024).

2.2 MICROBIOME ANALYSIS

Feces were collected from 3 piglets from each pen, on d 7, 14, 21 and 42. Samples were pooled per pen (n = 189 samples) and stored at -80°C until processing. All analyses were carried out at “Institut Universitaire de Cardiologie et Pneumologie de Québec” (IUCPQ, QC, Canada).

DNA was extracted using the QIAmp Power Fecal DNA kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations of the extracts were measured by Kit (Thermo Fisher Scientific MA, USA). The samples stored at -80°C until 16S rDNA library preparation according to the Illumina 16S ribosomal RNA gene V3-V4 region amplicon preparation protocol for the Illumina MiSeq System.

In summary, 12.5 ng of DNA was used as template, and the V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR; Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG. Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) in conjunction with the Nextera XT Index Kit V2 sets A and D (Illumina, CA, USA). The 16S metagenomic libraries were qualified with a Bioanalyser DNA 1000 Chip (Agilent, CA, USA) to verify the amplicon size (expected size ~630 bp) and quantified with a Qubit (Thermo Fisher Scientific, MA, USA). Libraries were then normalized and pooled to 4 nM and denatured and diluted to a final concentration of 8 pM. Sequencing was performed using the MiSeq Reagent KitV3 (600 cycles) on an Illumina MiSeq System (Illumina, CA, USA). Sequencing reads were generated in less than 65 h. Image analysis and base calling were carried out directly on the MiSeq.

Sequencing data were processed using the Divisive Amplicon Denoising Algorithm (DADA2) pipeline (CALLAHAN et al., 2016), and taxonomic assignation was done against the Silva v138 reference database. The operational taxonomic units that were present in fewer than three samples were filtered out.

Data analyses were performed in the web-based platform Microbiome Analyst 2.0 (LU et al., 2023). Data integrity and library size were checked. Data was filtered by low counter based on 10% prevalence in samples and removing 0% in low variance filter based on inter-quartile range. Normalization was performed by rarefaction to 4600 reads and then by total sum scaling and centered log ratio transformation. Univariate analysis, metagenome sequence, linear discriminant

analysis (LDA) effect size (LEfSe) using the Kruskal-Wallis sum rank test. Beta-diversity analysis was performed using the Bray-Curtis index of dissimilarity followed by permutational MANOVA and Analysis of group similarities (ANOSIM), and the ordination method was the principal coordinates analysis (PCoA). Alpha diversity measures were used to determine treatment effects on bacterial richness and evenness (i.e. Shannon, Simpon and Chao1 indexes) followed by Welch t-test/ANOVA. Significance was declared at $P < 0.05$. For visualization purposes, heat map clustering (Euclidean distance and the Ward clustering method), pie charts, and relative abundance bar plots were generated.

2.3 ANALYSIS OF PLASMA ENDOCANNABINOIDS AND OTHER LIPID MEDIATORS

Blood samples were collected from 3 piglets from each pen, on d 7, 14, 21 for the quantification of endocannabinoids mediators in the plasma (n = 144 samples). Plasma lipids were extracted according to (TURCOTTE et al., 2020) with some modifications. Briefly, 200 µL of plasma samples were mixed with 300 µL of TRIS-HCl (pH 7.4, 50 mM). Toluene (2 mL) containing 11.5 µl/ml acetic acid and 5 µl of internal standards were then added to the samples, vortexed for 1 minute, centrifuged at 4000g for 5 minutes with no brakes (2) at 4ºC. Samples then were placed in an ethanol-dry-ice bath (−80°C) to freeze the aqueous phase and the toluene upper phase was collected and evaporated to dryness under a stream of nitrogen. Samples were reconstituted in 58 µl of mobile phase containing 50% of solvent A (water + 1mM ammonium acetate + 0.05% acetic acid) and 50% of solvent B (acetonitrile/water95/5 + 1 mM ammonium acetate + 0.05% acetic acid). A 40 µL aliquot was injected onto an RP-HPLC column (Kinetex C8, 150 × 2.1 mm, 2.6 µm, Phenomenex). Quantification of eCBome-related mediators was carried out by liquid chromatography interfaced with the electrospray source of a Shimadzu 8050 triple quadrupole mass spectrometer and using multiple reactions monitoring in positive ion mode for the compounds and their deuterated homologs or a surrogate.

Quantification was achieved by generating calibration curves using pure standards and analyzed on the LC-MS/MS system three times. The slope was then

calculated using the ratio between the peak areas of the compound and its standard (1-AG-d5 for MAGs, AEA-d4 for anandamide or N-arachidonoyl-ethanolamine (AEA) and C17:1-LPA for the various LPA species).

The data was analyzed using the web-based platform MetaboAnalyst 5.0 (PANG et al., 2021). Non-filtered data were normalized by the sum method, generalized log-transformed, and Pareto-scaled. Multivariate analysis of data included partial least squares discriminant analysis (PLS-DA) using the Kruskal-Wallis sum rank test. Significance was declared at false discovery rate (FDR) < 0.05. For visualization purposes, heat maps were generated to showcase the magnitude of fold-change in a color gradient for increased (red) or decreased (blue) relative abundance.

2.4 OTHER STATISTICAL ANALYSES

Microbiome and endocannabinoid mediators' data found to be significantly affected by treatments where then analyzed in a mixed model in SAS 9.4 (SAS Institute, 2017) including pen as random effect and milk replacer, diet, time and the interactions as fixed effects. Significant differences were considered when $P < 0.05$ for main effects and $P < 0.10$ for interactions.

3 RESULTS

3.1 DIET AND MR EFFECTS ON TAXONOMY ASSIGNMENT

At the phylum-level, the relative abundance of *Firmicutes* (53.8%) was the highest, followed by *Bacteroidota* (38.4%) and *Spirochaetota* (2.5%) across all days and in groups. However, when observing the days individually (Figure 1A), there was variation between the phyla. *Bacteroidota* represented 45.6 and 46.6% of the total phyla, followed by *Firmicutes* with 45.5 and 46.3% on days 7 and 14, respectively. While on days 21 and 42, *Firmicutes* was more abundant, representing respectively, 64.3 and 57.6% of the total phyla.

Figure 1 - Relative abundance of different (A) Phylum and (B) Genus by time in piglets in the nursery phase.

Treatments were: 1) Milk Replacer: a commercial milk substitute rich in animal fat lipids and coconut oil (CO); a milk substitute rich in polar lipids (PO) or milk substitute rich in soy lipids (SO) until d 7; 2) Diet: solid diet rich in soy lipids (SD) or a diet rich in polar lipids from cow milk fat globular membranes (PD) from d 7 to d 21. From d 21 to 42 all piglets received a commercial diet.

A mixed-effects model analysis detected a three-way interaction between diet, MR, and time on the relative abundance of the *Proteobacteria* phylum ($P = 0.03$; Supplemental Table 1), whereas a tendency was observed for *Bacteroidota* ($P = 0.09$). Furthermore, there was a diet effect for *Desulfobacterota*, whose relative abundance was higher in the PD *relative to SD* dietary group *(*$P < 0.05$*)*, whereas a similar tendency was observed for *Actinobacteriota* ($P = 0.05$).

Most phyla were significantly affected by time. Indeed, many phyla showed significant time effects with *Actinobacteriota, Bacteroidota, Desulfobacterota, Proteobacteria, Spirochaetota* and *WPS-2* presenting an overall reduction in the dietary treatment period ($P < 0.01$), whereas *Campilobacterota, Cyanobacteria, Euryarchaeota, Firmicutes* and *Verrucomicrobiota* increased over that period ($P < 0.05$).

Prevotella, which belongs to the phylum *Bacteroidota,* was the genus with the highest relative abundance, representing 20.7% of the total of genera across treatments (Figure 1B). The next most abundant genera were *Lactobacillus* (10.4%) and *Agathobacter* (7.4%), both belonging to the *Firmicutes* phylum, whereas 14.5% were classified as *Not_Assigned*.

A mixed-effects model analysis detected a three-way interaction between diet, MR, and time on the relative abundance of the genera *Incertae_Sedis, Prevotella, Prevotellaceae_NK3B31_group* and *Succinivibrio* ($P <0.05$), whereas a tendency was observed for *Intestinimonas, Lachnospiraceae_NC2004_group, Lachnospiraceae_UCG_004, Prevotellaceae_UCG_003* and *UCG_005* ($P < 0.10$; Supplemental Table 2). A two-way interaction between time and diet was detected for the relative abundance of the genera *Alloprevotella, Anaerovibrio, Asteroleplasma, Campylobacter, Catenisphaera, Christensenellaceae_R_7_group, Desulfovibrio, Escherichia_Shigella, Horsej_a03, Lachnospiraceae_NK3A20_group, Lachnospiraceae_NK4B4_group, Lachnospiraceae_XPB1014_group, Megasphaera, Not_Assigned, Oribacterium, Oscillospira, possible_genus_Sk018, Prevotellaceae_UCG_004, Solobacterium, UCG_008* ($P < 0.05$). Whereas a tendency was observed for *Butyricicoccus, Candidatus_Soleaferrea, Family_XIII_AD3011_group, Lachnoclostridium,* and

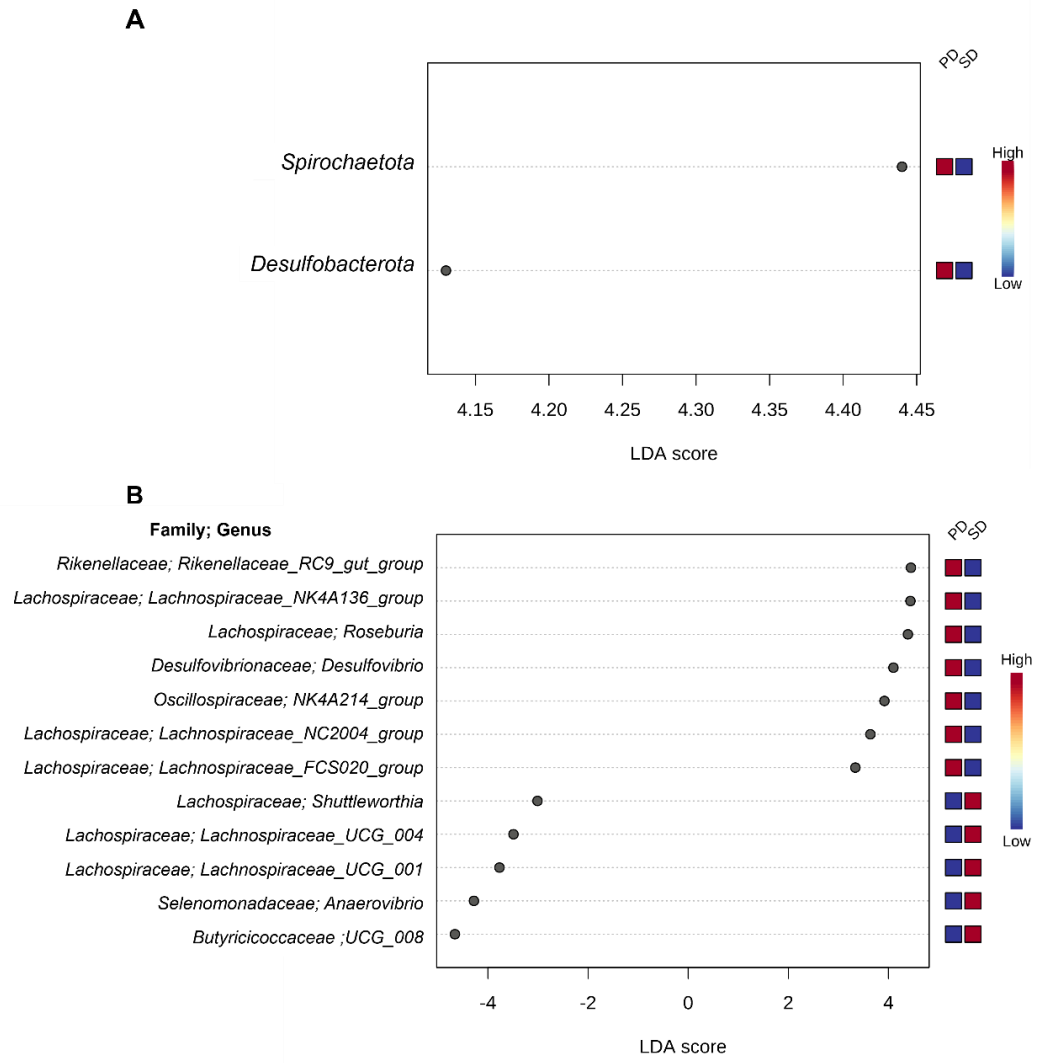
Parabacteroides ($P < 0.10$). An interaction between time and MR was detected only for the relative abundance of *Fusicatenibacter* ($P = 0.02$) whereas a tendency was observed for *Alloprevotella, Clostridium_sensu_stricto_6, Phascolarctobacterium* and *UCG_008* ($P <0.10$). While an interaction between MR and diet was detected only on the relative abundance of the *Agathobacter, Not_Assigned* ($P <0.05$) and a tendency to *Faecalibacterium* ($P =0.07$).

Most genera were significantly affected by time. Many genera showed significant time effects with *Agathobacter, Anaerovibrio, Bacteroides, CAG_873, Candidatus_Soleaferrea, Catenisphaera, Chlamydia, Christensenellaceae_R_7_group, Colidextribacter, Desulfovibrio, Escherichia_Shigella, Family_XIII_AD3011_group, Frisingicoccus, Mailhella, Monoglobus, Not_Assigned, Parabacteroides, Prevotellaceae_UCG_001, Prevotellaceae_UCG_004, Rikenellaceae_RC9_gut_group, Treponema, Turicibacter and UCG_002* having an overall reduction in the treatment period ($P < 0.01$). Whereas *Acidaminococcus, Asteroleplasma, Blautia, Butyricicoccus, Campylobacter, Catenibacterium, Clostridium_sensu_stricto_6, Dorea, Faecalibacterium, Family_XIII_UCG_001, Fusicatenibacter, Incertae_Sedis, Intestinibacter, Intestinimonas, Lachnospira, Lachnospiraceae_ND3007_group, Lachnospiraceae_NK3A20_group, Lachnospiraceae_NK4B4_group, Lachnospiraceae_UCG_004, Lactobacillus, Methanosphaera, Mitsuokella, Olsenella, Pseudobutyrivibrio, Ruminococcus, Shuttleworthia, Solobacterium, Subdoligranulum, Sutterella and Terrisporobacter* increased over that period ($P < 0.05$). The genera *Alloprevotella¸ Anaerostipes, Clostridium_sensu_stricto_1¸ Collinsella, Coprococcus, Denitrobacterium, Fournierella, Holdemanella, Horsej_a03, Lachnoclostridium, Lachnospiraceae_NC2004_group, Lachnospiraceae_NK4A136_group, Lachnospiraceae_UCG_001, Lachnospiraceae_XPB1014_group, Marvinbryantia, Megasphaera, NK4A214_group, Oribacterium, Oscillibacter, Oscillospira, Peptococcus, Phascolarctobacterium, possible_genus_Sk018, Prevotella, Prevotellaceae_NK3B31_group, Prevotellaceae_UCG_003, Selenomonas, Sphaerochaeta, Succinivibrio, UCG_003, UCG_004, UCG_005, UCG_008* varied

over time (P < 0.05). The genera *Coprococcus¸ NK4A214_group, Phascolarctobacterium and Roseburia,* were higher in the PD diet (P <0.05), while *Olsenella, Peptococcus and Rikenellaceae_RC9_gut_group* had a similar tendency (P <0.10). There was no impact of MR type on the relative abundance of any genus.

The Linear Discriminant Analysis Effect Size (LEfSe) showed a diet effect on the *Desulfobacterota* and *Spirochaetota* phyla, both of which were higher in the PD group (Figure 2A). Also, 12 genera were identified as characterizing PD and SD. *Rikenellaceae_RC9_gut_group*, *Lachnospiraceae_NK4A136_group, Roseburia, Desulfovibrio, Oscillospiraceae_NK4A214_group*, *Lachnospiraceae_NC2004_group, Lachnospiraceae_FCS020_group* were higher in PD diet with LDA score > 2. While *Shuttleworthia, Lachnospiraceae_UCG_004, Lachnospiraceae_UCG_001, Anaerovibrio* and, *UCG_008* were higher in SD treatment with LDA score < -2 (Figure 2B). No significant features were identified for MR at the feature-level, phylum or genus.

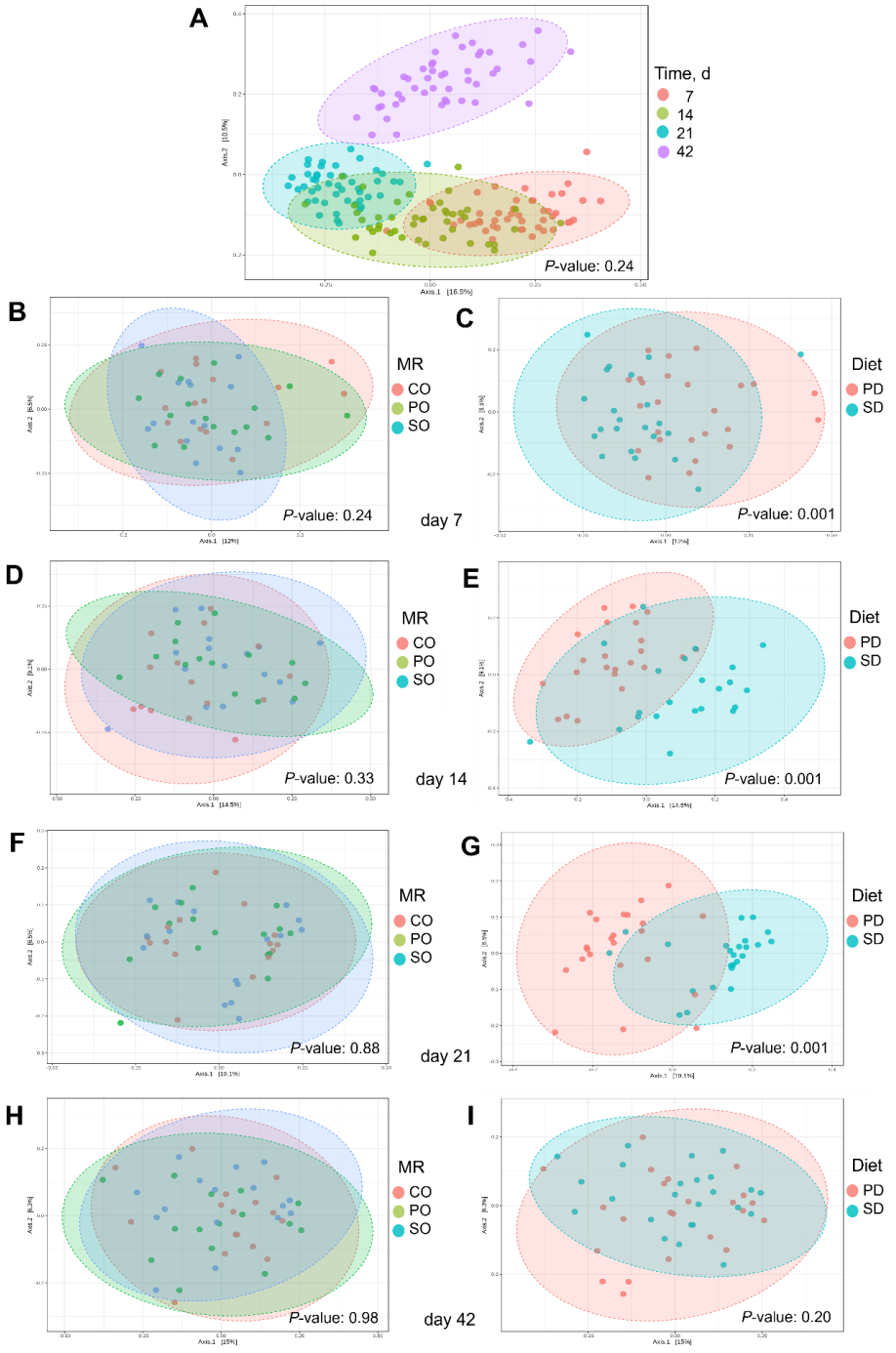
Figure 2 - Linear Discriminant Analysis Effect Size (LEfSe) at (A) Phylum and (B) Genus levels in the nursery phase in piglets fed a diet rich in soy lipids (SD) or a diet rich in polar lipids from cow milk fat globular membranes (PD).



3.2 BETA DIVERSITY

Likewise, the assessment of beta diversity by treatments demonstrated grouping by time at the feature-level, showing a clear progression of the development of the overall microbiome architecture over time (Figure 3). When analyzing sampling days individually, taxonomical distances were evident between the SD and PD dietary treatments on days 7 (P = 0.001), 14 (P = 0.001) and 21 (P = 0.001; Figures 3C, 3E and 3G, respectively).

Figure 3 - Principal coordinates analysis for Beta-diversity at the feature-level by time (A) a Milk Replacer (MR) and Diet from d 7 to 42 (B - I).

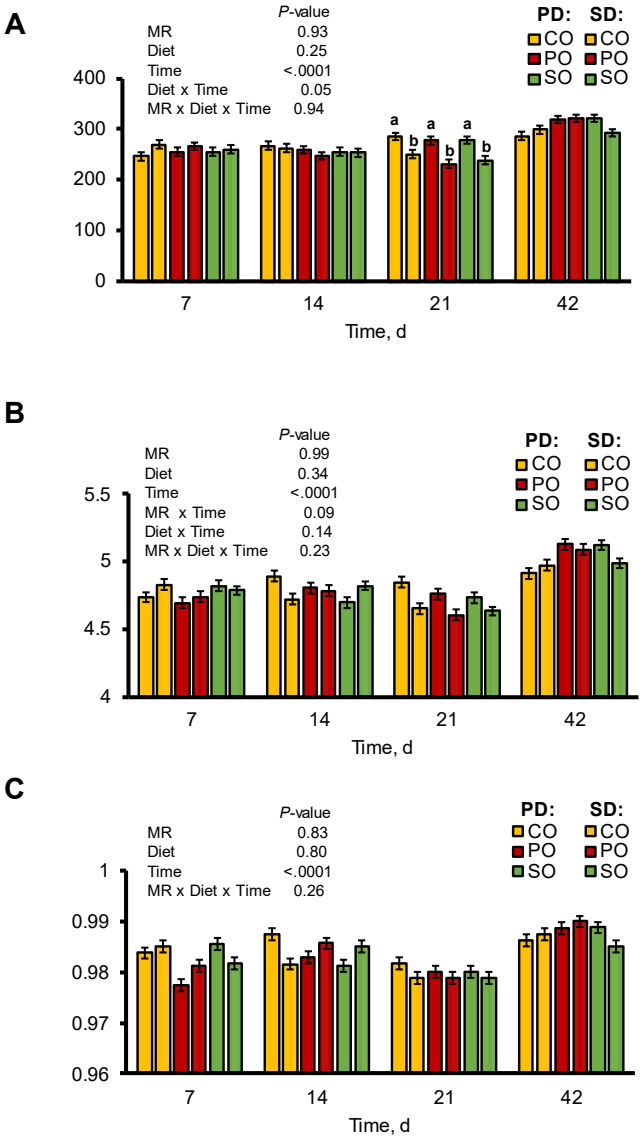


Treatments were: 1) Milk Replacer: commercial milk substitute rich in animal fat and coconut oil (CO); milk substitute rich in polar lipids (PO) or milk substitute rich in soy lipids (SO) until day 7; 2) Diet: solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD) from d 7 to d 21. From d 21 to 42 all piglets received a commercial diet.

3.3 ALPHA DIVERSITY

A mixed model analysis showed an effect of time ($P < 0.01$) on alpha diversity at resource level, whether evaluated by Chao1, Shannon or Simpson indexes (Figure 4; Supplemental Table 3). In addition, a tendency for a diet X time interaction for Chao-1, differences on d21 were explored, showing greater diversity in PD relative to SD irrespective MR type ($P = 0.05$). No differences were observed between groups according to MR type.

Figure 4 - Alpha diversity at feature level by time analyzed by (A) CHAO1, (B) SHANNON and (C) SIMPSON.



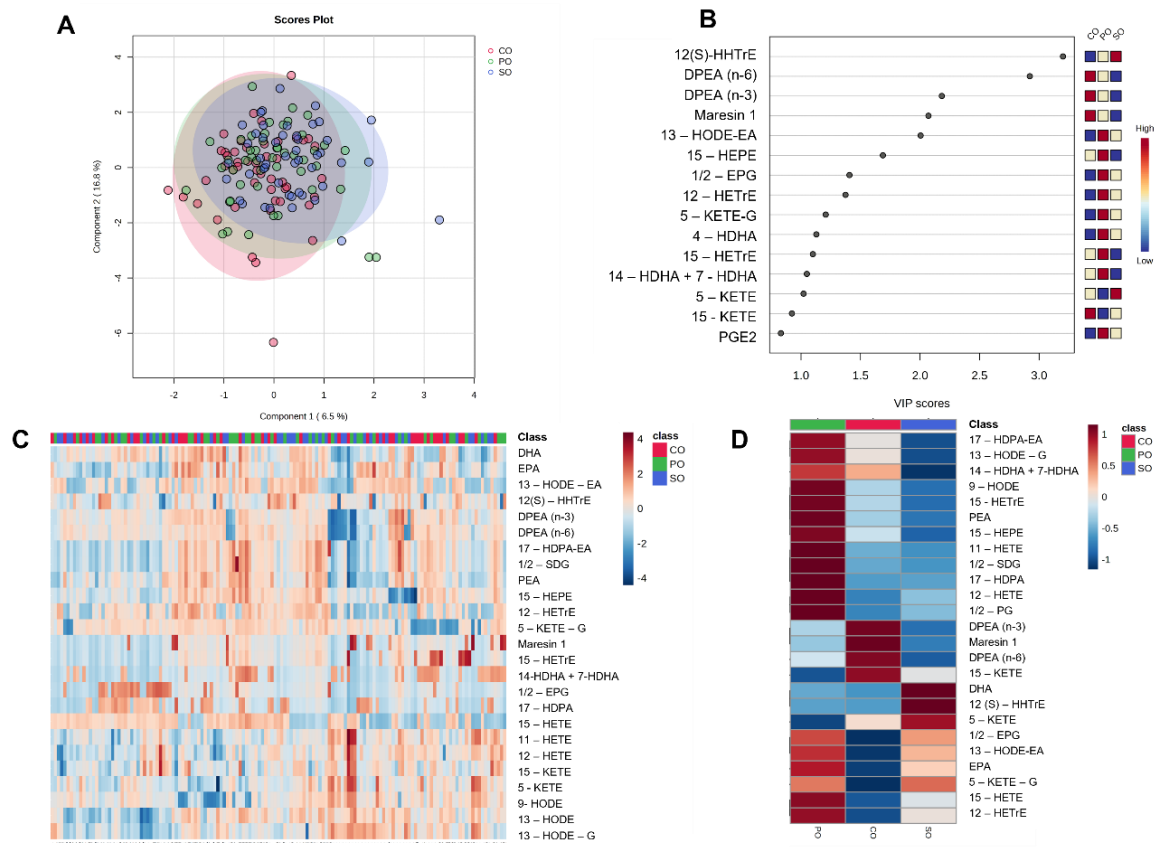
Treatments were: 1) Milk Replacer: a commercial milk substitute rich in animal fat lipids and coconut oil (CO); a milk substitute rich in polar lipids (PO) or milk substitute rich in soy lipids (SO) until day 7; 2) Diet: solid diet rich in soy lipids (SD) or a diet rich in polar lipids from cow milk fat globular membranes (PD) from d 7 to d 21. From d 21 to 42 all piglets received a commercial diet. Different letters indicate statistical difference (<0.05) between diets on the same day of analysis.

3.4 ENDOCANNABINOIDS AND LIPID MEDIATORS

Two-factor analysis revealed no interaction between milk replacer and diet for plasma lipids ($P > 0.05$). The factors were then analyzed individually.

Partial least squares discriminant analysis (PLS-DA) plots of the plasma endocannabinoidome and other lipid mediators' data revealed unique features predictive of CO, PO and SO MR treatments (**Figure 5A & B**). The VIP scores analysis showed that DEPEA n-6, DEPEA n-3 and Maresin 1 had a VIP score > 1 and were highest in the CO MR treatment. 13 – HODE-EA, 15 – HEPE, 1/2 – EPG, 12 – HETrE, 5 – KETE-G, 4-HDHA, 15-HETrE, 14-HDHA + 7 – HDHA and PGE_2 had VIP scores > 1 and were highest in the PD MR treatment. While 12(S)-HHTrE and 5-KETE had VIP scores > 1 and were highest in the SO MR treatment (**Figure 5B**). Heat mapping after hierarchical clustering revealed no clear clustering of endocannabinoidome and other lipid mediators type concentrations by MR (**Figure 5C**). However, when adjusted by treatment (**Figure 5D**) the heatmap showed a high concentration of the lipid mediators DPEA (n-3), Maresin 1, DPEA (n-6) and 15 – KETE were highest in CO. Lipid mediators 17 – HDPA-EA, 13 – HODE – G, 14 – HDHA + 7-HDHA, 9 – HODE, 15 – HETrE, PEA, 15 – HEPE, 11 – HETE, 1/2 – ODS, 17 – HDPA , 12 – HETE, 1/2 – PG, 15 – HETE and 12 – HETrE were higher in PO. DHA, 12 (S) – HHTrE, 5 – KETE were higher in SO. While 1/2 – EPG, 13 – HODE-EA, EPA and 5 – KETE – G were concentrated in PO and SO.

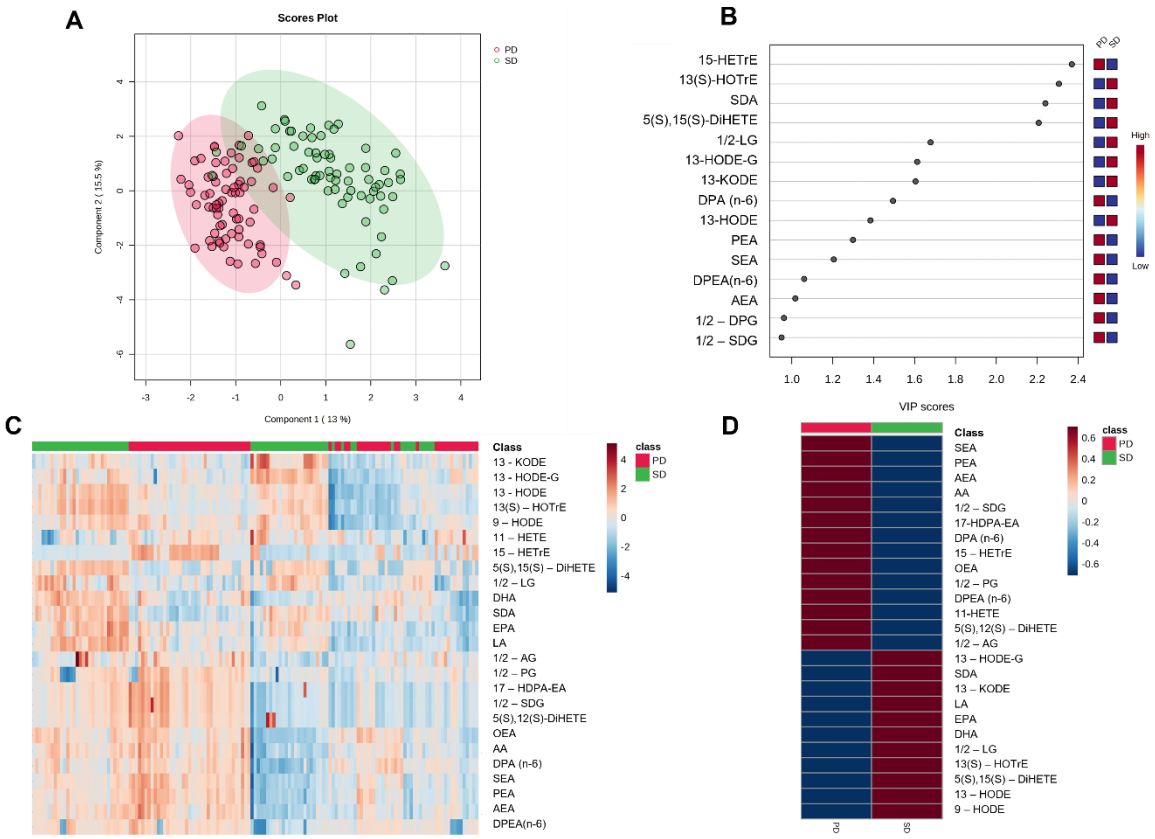
Figure 5 - Partial least squares discriminant analysis of endocannabinoids and lipid mediators in plasma on day 7 of the nursery phase in piglets fed a commercial milk substitute rich in animal fat lipids and coconut oil (CO); a milk substitute rich in polar lipids (PO) or milk substitute rich in soy lipids (SO). **A:** two-dimensional partial least squares discriminant (PLS-DA) score plot. **B:** variable importance projection (VIP) scores analysis based on component 1 of the PLS-DA used to rank the relative contribution of lipids to the variance between treatments. **C:** heatmap clustering analysis of the plasma endocannabinoids and lipid mediators influenced by milk replacer treatments. **D:** heatmap group averages.



Plasma endocannabinoids and lipids mediators data were obtained using a LC–MS/MS custom assay.

Differently, the PLS-DA plots of the plasma endocannabinoidome and other lipid mediators' data revealed a clear clustering of PD and SD diet treatments on d7 of treatment (**Figure 6A**). The variable importance projection (VIP) scores analysis showed that 15-HETrE, DPA (n-6), PEA, SEA, DPEA (n-6) and AEA were predictive of the PD treatment (VIP score >1), while 13(S)-HOTrE, SDA, 5(S),15(S)-DiHETE, 1/2 – LG, 13 – HODE-G, 13 – KODE and 13 – HODE were predictive of the SD treatment. The heatmap revealed a clear clustering of endocannabinoid and lipid mediators by diet (**Figure 6C**). The heatmap of group averages (**Figure 6D**) showed a high concentration of the lipid mediators SEA, PEA, AEA, AA, 1/2 – SDG, 17-HDPA-EA, DPA n-6, 15 – HETrE, OEA, 1/2 – PG, DPEA n-6, 11-HETE, 5(S),12(S) – DiHETE and 1/2 – AG in PD. While 13 – HODE-G, SDA, 13 – KODE, LA, EPA, DHA, 1/2 – LG, 13(S) – HOTrE, 5(S),15(S) – DiHETE, 13 – HODE and 9 – HODE were higher in SD.

Figure 6 **-** Partial least squares discriminant analysis and hierarchical grouping of endocannabinoids and lipid mediators in plasma on day 7 of the nursery phase in piglets fed a diet rich in soy lipids (SD) or a diet rich in polar lipids from cow milk fat globular membranes (PD). **A:** two-dimensional partial least squares discriminant (PLS-DA) score plot. **B:** variable importance projection (VIP) scores analysis based on component 1 of the PLS-DA used to rank the relative contribution of lipids to the variance between treatments. **C:** heatmap clustering analysis of the plasma endocannabinoids and lipid mediators influenced by milk replacer treatments. **D:** heatmap group averages.



Plasma endocannabinoids and lipids mediators data were obtained using a LC–MS/MS custom assay.

4 DISCUSSION

Stress due to separation from the sow, the new environment and the abrupt transition from liquid to solid diet during the weaning period causes changes in the intestinal structure of piglets (MOESER; POHL; RAJPUT, 2017), dysbiosis in gut microbiota (WEI et al., 2021), and can trigger inflammatory processes (NORRIS et al., 2017; CAMPBELL et al., 2013). Our model of weaning stress used milk replacers seven days after weaning to reduce oxidative stress related to food adaptation and intestinal maturation, in addition to testing different sources of dietary lipids in milk replacers as well as part of the solid diet up to 21 days after weaning.

The expectation was that animals that received polar lipids, either in the milk replacer or in the diet - coming from a source of polar lipids from a modified by-product of cheese manufacturing rich in sphingolipids - would present lower levels of markers of immune activation and gut permeability (i.e., plasma lipopolysaccharide binding protein (LBP) and fecal calprotectin; Bischoff et al., 2014; Seethaler et al., 2021). Importantly, immune responses are also mediated through a wide range of lipid species (i.e., oxylipids and endocannabinoids), which in turn are associated with intestinal microbiota composition (MUCCIOLI et al., 2010). However, polar lipid-receiving groups did not exhibit changes in LBP and Calprotectin relative to animals in the soy lipid groups (Chakroun et al., Unpublished). It is likely that lack of differences between groups was related to the use of a mild weaning stress model, which allowed animals for a smooth and progressive transition into the introduction of solid feed. Indeed, weaning stress models not including access to liquid feed are known to cause low feed intake, increased intestinal permeability and this immune activation (DONG; PLUSKE, 2007; UPADHAYA; KIM, 2021).

However, although there was no difference in the above-mentioned inflammatory mediators, our lipidomic data (LARSEN et al., 2024) demonstrated that the lipid profile of dietary treatments (PD and SD) had a great influence on the plasma lipid composition of piglets at 7 days post-weaning, whereas no major changes were observed in response lipids contained in the MR treatments (CO, PO and SO). As the lipids present in the diet influence the composition of the microbiota

and this is related to inflammatory processes in the gastrointestinal tract (SCHOELER; CAESAR, 2019), we aimed in the present study to investigate changes in the composition of the microbiota and lipid mediators from day 7 to day 42 of the experiment in the different dietary treatments. To our knowledge, our study is the first to evaluate the influence of dietary polar lipids on both plasma lipid mediators and intestinal microbiota composition of weaned piglets.

Firmicutes and *Bacteriodota* are the predominant phyla in the swine gut microbiota, followed by *Proteobacteria, Actinobacteria,* and *Spirochaetota* (WEI et al., 2021), which corroborates our findings. Although this predominance is generally stable, diet and age can influence the overall composition of the microbiota, in particular at lower levels of taxonomic classification. Our study demonstrated similar behavior at the phylum and genus level over time. Most of the genera that decreased their relative abundance belong to the phyla *Actionobacteriota, Bacteroidota, Protobacteria, Spirochaetota*, and these phyla also decreased their relative abundance over time. While most of the genera that increased over time belong to the phylum *Firmicutes*, which had the same effect. The genera that varied over time mainly belong to the phyla *Bacteriodota* and *Firmicutes*. Corroborating our findings, (NORRIS et al., 2016) reported that sphingomyelin from dietary milk modulates fecal microbiota composition in mice, increasing *Firmicutes* and reducing the relative abundance of *Bacteroidota*. According to Rohrhofer et al. (2021), an increase in the concentration of dietary sphingolipids induces changes in intestinal microbiota composition, since Sphingosine and lyso-SLs have antimicrobial properties and compete with commensal bacteria for attachment to intestinal cells to prevent pathogen invasion. Although the main source of variation in plasma SL is expected to be dietary levels of these compounds, some SL are also synthesized by gut bacteria (NORRIS et al., 2017). Sphingolipids are produced by bacteria of the phylum *Bacteroidota (Bacteroides, Prevotella, Porphyromonas, Sphingobacterium)* and by *Proteobacteria (Sphingomonas, Bdellovibrio, Acetobacter*), depending on the abundance of these phyla in the gut microbiota (OLSEN; JANTZEN, 2001).

Prevotella was the predominant genus, representing 20.7% of the total of genera among treatments on day 7 post-weaning and had an interaction effect of

diet, MR and time. Similarly, Wei et al. (2021) found that the relative abundance of the genus *Prevotella* increased by 2-fold between the day of weaning to day 7 post-weaning after the introduction of solid plant-based diets. While Heinritz et al. (2016) found a higher prevalence of *Prevotella* when testing a high-fat/low-fiber diet (249 g fat/kg DM) based on lipids from sunflower margarine, sweet cream butter, and soybean oil, compared to a standard diet containing 30 g fat/kg DM in three-month-old pigs. Other genera such as *Megasphaera* and *Blautia*, which are involved in carbohydrate degradation, also increased during the post-weaning period in the study by Wei et al. (2021). In our study, *Blautia* showed a significant increase in abundance until day 21 and decreased on day 42 but was not influenced by treatment, whereas *Megasphaera* abundance was higher in PD on days 7, 14 and 42, and lower on day 21, showing a time-dependent diet effect. In summary, diet had an impact on the diversity and composition of the microbiota, which for several taxa was time-dependent, probably reflecting progressive adaptation to dietary treatments.

Discriminant analysis indicated that most of the genera found in the LEfSe analysis were from the family *Lachnospiraceae*, producers of short-chain fatty acids (SCFA) that consume carbohydrates and have anti-inflammatory properties (FUSCO et al., 2023; XU et al., 2021). This result demonstrated that this family is particularly sensitive to the types of dietary lipids and, therefore, its abundance and function can be altered by diet. PD treatment showed an increase in *Coprococcus, Roseburia* (from *Lachnospiraceae family)* and *Phascolarctobacterium* (from the *Acidaminococcaceae* family; another producer of SCFA). In contrast, the pro-inflammatory genus *Desulfovibrio* was also indicative of PD. For SD treatment, LEfSe demonstrated *Butyricicoccaceae; UCG-008* and *Anaerovibrio* as indicatives that have been associated with inflammatory diseases in other studies (AMEER et al., 2023; LI et al., 2022). Our study did not demonstrate differences in inflammatory markers, however, considering a more stressful weaning situation, these changes in the microbiota sensitive to dietary lipids may influence susceptibility to inflammation.

Furthermore, Yang et al. (2020) proposed that in addition to the impact of having diverse lipid types, fat digestibility also influenced the composition of the

microbiota of piglets in the nursery when comparing soybean oil, palm oil, and encapsulated palm oil. Our analysis reveals that, although at equal energy levels, the treatment lipid sources and their compositions differed (LARSEN et al., 2024). The solid polar lipid diet (PD) contained 31-fold more total SL compared with the soybean-based diet (SD). Whereas the polar lipid MR (PO) contained 40-fold and 2-fold more total sphingolipids relative to the soybean (SO) and the control (CO) groups. From this, it was expected that the changes in the profile of plasma lipid mediators would be influenced by the dietary fatty acid profile, as suggested by Bourdeau-Julien et al. (2023). Interestingly, the polar lipid diet mostly presented enrichment in lipid mediators whose precursors are dihomo-g-linolenic acid (DGLA; for 15-HETrE), arachidonic acid (AA; for AEA), and docosapentaenoic acid from the n-6 pathway (DPA n-6; for DPEA n-6 and 1/2 – DPG). All of these lipids can also be derived from linoleic acid (LA; SIMARD et al., 2022), however, it only represented 23% of the total FA in the PD group, whereas it was two times higher in the SD group. This suggests that the PD diet resulted in increased levels of these lipid mediators, likely by alterations in enzyme activity rather than direct precursor availability.

Furthermore, pigs in the PD treatment exhibited higher concentrations of the lipid mediators PEA and SEA, derived from palmitic and stearic acids, respectively (HANSEN, H. S.; , KLEBERG, K.,; HASSING, H. A., 2015; KEPPEL HESSELINK; KOPSKY; WITKAMP, 2014). Importantly, reduced feed intake was observed in the PD group (Chakroun et al., Unpublished), which could be associated to the increased concentrations of PEA, a lipid mediator previously shown to reduced feed intake and inflammation via (PPARA and TRPV1; BRANKOVIĆ et al., 2024) Palmitic and stearic acids represented, respectively, 27 and 8% of total FA in the dietary composition of the PD diet. On the other hand, pigs in the SD group exhibited higher concentrations of lipid mediators whose precursors are LA (5(S),15(S)-DiHETE, 1/2-LG, 13-HODE-G, 13-KODE and 13-HODE) and alpha-linolenic acid (ALA for 13(S)-HOTrE and SDA), the latter being 4.6 times higher in the SD compared to the PD diet.

Milk replacer discriminant features were lipid mediators whose precursors are DGLA (12 – HETrE, 15 – HETrE), AA (15 - KETE, PGE2 and 5 – KETE), DPA n-6 (DPEA n-6) and LA (13 – HODE-EA, 12(S)-HHTrE), all from LA pathway (SIMARD et al., 2022). Probably arising from the presence of AA and, in greater quantities, LA (7 to 14% of the total FA across MR groups). Lipid mediators whose precursors are docosahexaenoic acid (DHA; 4 – HDHA and 14 – HDHA + 7 – HDHA), DPA n-3 (DPEA n-3), EPA (15 – HEPE and 1/2 – EPG) and eicosatetraenoic acid (ETA; 5 – KETE-G) are from the ALA pathway (SIMARD et. al, 2022). DHA and DPA from n-3 pathway were identified in low quantities in the three treatments lipid composition (approximately 0.05 and 0.07% of total FA, respectively) while EPA and ETA were not identified in diet. However, their main precursor ALA represented 0.43, 0.39 and 1.05% of total FA in CO, PO and SO, respectively. In addition, Maresin 1, a bioactive molecule belonging to the family of specialized pro-resolving lipid mediators (SPMs) whose precursor is DHA (FERREIRA et al., 2022) was identified with the highest concentration in CO. The fact that the majority of lipid mediators affected by treatment are classified as anti-inflammatory agents, regardless of whether they come from the n-3 or n-6 pathway, supports the idea that our model of weaning stress (i.e., providing MR for the first week) may have precluded the synthesis of pro inflammatory mediators.

AA-derived molecules AEA and 2-AG exhibited higher concentrations in the plasma of piglets fed the PD diet. Both lipids are endogenous ligands for the CB_1 and CB_2 receptors of the endocannabinoid (eCB) system (VEILLEUX; DI MARZO; SILVESTRI, 2019). This suggests a greater activation of this system in the treatment of PD compared to SD. Importantly, the CB_1 receptor can reduce intestinal permeability and plasma LPS (CANI et al., 2007). Furthermore, blocking the CB_1 receptor influences the reduction of inflammation (MUCCIOLI et al., 2010). However, as already mentioned, we did not observe any effects of treatments on markers of intestinal permeability or inflammation. Nonetheless, given that the eCB system tone is associated with intestinal microbiota composition through the modulation of CB_1 and FAAH expression (ROUSSEAUX et al., 2007), we analyzed possible changes in microbiota related to the eCB receptors and their ligand lipids AEA and 2- AG.

AEA is able to reverse adverse disturbances in the microbiota in mice with respiratory syndrome, increasing the abundance of beneficial SCFA-producing bacteria, restricting inflammation and reducing *Enterobacteriaceae* - belonging to the phylum *Proteobacteria* - and pathogenic *Pseudomonas* in the lungs of mice receiving AEA treatment (SULTAN et al., 2021). The same authors also reported an increase in the relative abundance of *Lachnospiraceae* and *Clostridia* - family and class belonging to the phylum *Firmicutes*. In our study, the phyla *Firmicutes* and *Proteobacteria* were only affected by time.

5 CONCLUSION

Providing polar lipids in the MR for the first 7 days had little impact on overall microbiota composition but influenced specific plasma lipid mediators, particularly those derived from DGLA, AA, and LA. The inclusion of polar lipids in the diet until day 21 significantly altered microbiota composition, favoring an increase in specific genera from the *Lachnospiraceae* family (e.g., *Coprococcus* and *Roseburia*) and other *Firmicutes*-associated taxa, which are known for their potential anti-inflammatory properties. However, some pro-inflammatory genera, such as *Desulfovibrio*, were also enriched in PD. Time on diet played a critical role in shaping microbiota dynamics.

Additionally, the PD diet increased levels of endocannabinoids, such as AEA and 2-AG, which are involved in gut barrier function and immune regulation, although no direct improvements in gut permeability markers were observed. In contrast, the SD was associated with higher levels of pro-inflammatory lipid mediators, such as 13-HODE and 13-KODE, derived from linoleic acid.

Although no immediate inflammatory responses were detected through conventional biomarkers, the observed microbiota and lipidomic shifts suggest a potential long-term impact on immune regulation and metabolism. Future studies should explore the effects of polar lipid supplementation under more stressful weaning conditions, as more severe weaning stress models might better reveal the potential of these lipids in mitigating inflammation and improving piglet adaptation.

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SUPPLEMENTAL TABLES

Supplemental Table 1. Effects of milk replacers (MR) and diets on relative abundance of different phylum in piglets on the nursery phase.

		PD			SD				*P*-value						
Phylum	Time	CO	PO	SO	CO	PO	SO	SEM	MR	Diet	Time	MR*Diet	Time*MR	Time*Diet	MR*Diet*Time
Actinobacteriota	7	0.41	0.59	0.14	0.28	0.23	0.13	0.16	0.11	0.05	<0.0001	0.30	0.40	0.58	0.96
	14	0.19	0.14	0.12	0.11	0.12	0.16	0.04							
	21	0.39	0.35	0.20	0.24	0.29	0.22	0.07							
	42	0.33	0.42	0.31	0.26	0.28	0.30	0.09							
Bacteroidota	7	44.0	48.8	44.4	45.6	43.5	48.3	1.92	0.11	0.41	<0.0001	0.08	0.94	0.52	0.09
	14	41.4	49.1	46.5	48.1	49.3	46.1	3.00							
	21	28.1	32.3	29.7	32.2	30.0	31.5	1.31							
	42	32.2	31.2	32.8	30.8	33.2	29.4	1.63							
Campilobacterota	7	0.17	0.16	0.31	0.33	0.24	0.50	0.15	0.18	0.71	0.03	0.34	0.29	0.06	0.61
	14	0.32	0.56	0.18	0.54	0.63	0.56	0.20							
	21	0.59	0.59	0.47	0.40	0.48	0.35	0.12							
	42	0.29	1.24	0.77	0.52	0.54	0.21	0.23							
Cyanobacteria	7	0.09	0.03	0.35	0.04	0.21	0.50	0.20	0.13	0.68	<0.0001	0.93	0.42	0.47	0.48
	14	1.06	0.62	1.90	0.52	1.09	2.11	0.62							
	21	1.15	2.22	1.14	1.33	0.79	0.86	0.41							
	42	0.85	0.64	1.12	0.68	1.16	1.05	0.22							
Desulfobacterota	7	1.25	1.52	1.18	1.26	0.80	1.05	0.24	0.70	0.002	<0.0001	0.05	0.35	0.27	0.59
	14	0.91	1.14	1.21	0.82	0.42	0.78	0.22							
	21	0.71	0.75	0.68	0.27	0.34	0.35	0.12							
	42	0.80	1.59	0.83	0.98	1.12	1.06	0.18							
Euryarchaeota	7	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.21	0.32	<0.0001	0.15	0.17	0.17	0.35
	14	0.03	0.01	0.03	0.03	0.00	0.05	0.08							
	21	0.32	0.30	0.11	0.20	0.04	0.04	0.08							
	42	0.74	0.51	0.54	0.57	0.49	0.83	0.08							
Fibrobacterota	7	0.05	0.14	0.12	0.22	0.10	0.05	0.06	0.94	0.73	0.16	0.59	0.98	0.71	0.22
	14	0.06	0.10	0.05	0.08	0.04	0.11	0.06							
	21	0.10	0.12	0.02	0.01	0.01	0.06	0.06							
	42	0.16	0.09	0.13	0.08	0.13	0.15	0.06							

Firmicutes	7	44.6	42.3	47.1	47.1	47.5	43.7	2.14	0.38	0.76	<0.0001	0.13	0.98	0.52	0.51
	14	51.2	44.5	46.5	45.2	45.0	45.3	2.43							
	21	66.1	61.4	65.0	63.4	66.6	65.2	2.27							
	42	58.7	56.0	56.4	57.3	57.2	59.9	2.27							
Patescibacteria	7	0.06	0.27	0.02	0.01	0.05	0.05	0.05	0.25	0.28	0.28	0.40	0.28	0.25	0.50
	14	0.01	0.10	0.02	0.00	0.04	0.04	0.05							
	21	0.09	0.04	0.01	0.02	0.02	0.01	0.05							
	42	0.04	0.07	0.03	0.03	0.08	0.06	0.05							
Proteobacteria	7	4.17	2.00	1.86	1.46	4.51	2.63	0.66	0.62	0.18	0.001	0.32	0.82	0.17	0.03
	14	1.35	1.32	1.49	2.92	2.73	2.81	0.70							
	21	1.10	1.00	1.48	1.38	1.31	1.25	0.66							
	42	1.54	1.89	1.43	2.03	1.78	0.81	0.66							
Spirochaetota	7	3.82	3.35	3.42	3.16	2.00	3.09	0.80	0.62	0.14	<0.0001	0.36	0.93	0.56	0.22
	14	3.12	2.22	2.23	1.53	0.84	1.70	0.85							
	21	0.78	0.63	0.98	0.27	0.19	0.19	0.80							
	42	3.37	6.00	5.09	5.73	3.42	5.52	0.80							
Verrucomicrobiota	7	0.07	0.09	0.14	0.00	0.10	0.01	0.08	0.16	0.45	<0.0001	0.25	0.32	0.14	0.21
	14	0.01	0.07	0.02	0.01	0.40	0.03	0.09							
	21	0.10	0.11	0.16	0.00	0.03	0.01	0.08							
	42	0.29	0.42	0.39	0.46	0.26	0.26	0.08							
WPS-2	7	1.39	0.79	0.96	0.59	0.78	0.25	0.28	0.29	0.15	0.002	0.83	0.75	0.29	0.87
	14	0.37	0.36	0.27	0.05	0.01	0.28	0.30							
	21	0.16	0.17	0.02	0.27	0.00	0.02	0.28							
	42	0.68	0.31	0.14	0.62	0.32	0.46	0.28							

Treatments were: 1) Milk Replacer: commercial milk substitute rich in animal fat and coconut oil (CO); milk substitute rich in polar lipids (PO) or milk substitute rich in soy lipids (SO) until day 7; 2) Diet: solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD) from d 7 to d 21. From d 21 to 42 all piglets received a commercial diet.

Supplemental Table 2 – Effects of milk replacers (MR) and diets on relative abundance of different in piglets on the nursery phase.

Genus	Time	PD			SD			SEM	*P*-value						
		CO	PO	SO	CO	PO	SO		MR	Diet	Time	MR* Diet	Time*MR	Time*Diet	MR* Diet* Time
Acidaminococcus	7	0.09	0.00	0.00	0.03	0.00	0.00	0.04	0.73	0.46	<0.0001	0.62	0.6	0.16	0.73
	14	0.02	0.02	0.05	0.01	0.00	0.02	0.02							
	21	0.13	0.18	0.14	0.24	0.20	0.37	0.07							
	42	0.20	0.20	0.20	0.12	0.23	0.24	0.10							
Agathobacter	7	3.97	2.40	5.17	5.08	8.00	6.94	1.29	0.94	0.04	<0.0001	0.03	0.67	0.28	0.89
	14	6.53	5.39	6.72	6.60	7.10	7.91	1.17							
	21	9.48	8.68	9.07	9.48	11.01	9.08	0.81							
	42	9.81	6.14	7.32	7.09	8.72	7.04	1.40							
Alloprevotella	7	1.10	1.15	1.30	1.89	1.62	3.17	0.33	0.9	0	<0.0001	0.88	0.09	<0.0001	0.26
	14	1.43	0.96	0.67	2.41	2.46	2.29	0.43							
	21	0.27	0.54	0.84	0.62	0.64	0.57	0.21							
	42	1.51	1.83	1.56	1.30	1.51	0.73	0.32							
Anaerostipes	7	0.10	0.00	0.00	0.03	0.00	0.05	0.03	0.47	0.2	0.03	0.59	0.63	0.66	0.55
	14	0.21	0.04	0.06	0.06	0.18	0.08	0.10							
	21	0.09	0.05	0.26	0.11	0.04	0.11	0.08							
	42	0.29	0.10	0.27	0.13	0.17	0.04	0.14							
Anaerovibrio	7	2.26	1.67	1.53	2.34	2.90	2.69	0.48	0.96	0.03	0.003	0.11	0.96	0.01	0.29
	14	2.16	1.62	1.79	2.55	3.54	3.11	0.45							
	21	1.79	1.62	1.52	2.00	1.63	1.85	0.24							

	42	2.56	1.74	3.25	1.91	2.34	1.54	0.57							
Asteroleplasma	7	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.59	0.02	<0.0001	0.92	0.96	0.02	0.97
	14	0.00	0.00	0.00	0.01	0.00	0.00	0.06							
	21	0.04	0.01	0.00	0.05	0.04	0.06	0.06							
	42	0.17	0.09	0.19	0.41	0.31	0.30	0.06							
Bacteroides	7	0.72	1.17	1.16	0.66	1.31	0.83	0.43	0.75	0.49	<0.0001	0.7	0.59	0.82	1
	14	0.16	0.24	0.76	0.08	-0.01	0.14	0.43							
	21	0.02	0.00	0.56	0.01	0.00	-0.07	0.43							
	42	0.01	0.01	0.59	0.04	0.01	-0.06	0.43							
Blautia	7	0.67	0.56	0.87	1.16	0.94	0.72	0.34	0.62	0.14	<0.0001	0.18	0.7	0.22	0.42
	14	1.21	1.09	0.79	1.22	1.00	1.09	0.36							
	21	3.10	2.50	3.03	2.87	3.85	3.89	0.34							
	42	2.05	1.66	2.27	1.42	1.89	1.94	0.34							
Butyricicoccus	7	0.00	0.00	0.00	0.01	0.01	0.03	0.02	0.92	0.04	<0.0001	0.04	0.93	0.05	0.19
	14	0.01	0.01	0.01	0.03	0.04	0.02	0.02							
	21	0.13	0.07	0.11	0.13	0.20	0.17	0.02							
	42	0.07	0.01	0.03	0.01	0.08	0.01	0.02							
CAG_873	7	0.16	0.21	0.14	0.20	0.14	0.01	0.05	0.65	0.15	0.0003	0.54	0.65	0.22	0.75
	14	0.08	0.18	0.11	0.04	0.00	0.01	0.05							
	21	0.03	0.04	0.01	0.00	0.01	0.05	0.05							
	42	0.01	0.01	0.00	0.02	0.01	0.06	0.05							
Campylobacter	7	0.17	0.16	0.31	0.33	0.24	0.51	0.18	0.23	0.75	0.03	0.39	0.23	0.02	0.36
	14	0.32	0.55	0.19	0.55	0.62	0.56	0.19							
	21	0.59	0.59	0.46	0.40	0.48	0.35	0.18							
	42	0.29	1.22	0.77	0.52	0.54	0.22	0.18							
	7	0.06	0.27	0.02	0.01	0.05	0.05	0.05	0.25	0.28	0.28	0.4	0.28	0.25	0.5

Candidatus_Saccharimonas	14	0.01	0.10	0.02	0.00	0.04	0.04	0.05							
	21	0.09	0.04	0.01	0.02	0.02	0.01	0.05							
	42	0.04	0.07	0.03	0.03	0.08	0.06	0.05							
Candidatus_Soleaferrea	7	0.13	0.03	0.03	0.01	0.01	0.00	0.03	0.4	0.14	<0.0001	0.45	0.19	0.05	0.13
	14	0.06	0.02	0.04	0.02	0.00	0.01	0.02							
	21	0.04	0.02	0.02	0.03	0.03	0.01	0.01							
	42	0.04	0.12	0.09	0.11	0.07	0.17	0.03							
Catenibacterium	7	0.03	0.00	0.00	0.00	0.00	0.00	0.06	0.57	0.12	0.0007	0.69	0.65	0.71	0.93
	14	0.25	0.06	0.04	0.04	0.01	0.01	0.07							
	21	0.21	0.15	0.15	0.15	0.13	0.15	0.06							
	42	0.10	0.13	0.19	0.08	0.11	0.10	0.06							
Catenisphaera	7	0.15	0.34	0.16	0.20	0.16	0.07	0.06	0.23	0.67	<0.0001	0.32	0.16	0.002	0.59
	14	0.13	0.12	0.05	0.03	0.06	0.02	0.07							
	21	0.13	0.28	0.17	0.06	0.18	0.10	0.06							
	42	0.21	0.18	0.16	0.33	0.26	0.46	0.06							
Chlamydia	7	0.02	0.00	0.12	0.00	0.08	0.02	0.07	0.14	0.77	0.0001	0.32	0.19	0.2	0.38
	14	0.00	0.07	0.01	0.01	0.39	0.03	0.07							
	21	0.00	0.00	0.02	0.00	0.01	0.00	0.07							
	42	0.18	0.27	0.21	0.19	0.12	0.16	0.07							
Christensenellaceae_R_7_group	7	2.40	1.00	0.96	0.74	0.51	0.54	0.63	0.56	0.11	<0.0001	0.36	0.72	0.002	0.84
	14	0.37	0.48	0.32	0.19	0.09	0.21	0.11							
	21	0.57	0.55	0.41	0.20	0.11	0.32	0.15							
	42	0.43	0.83	0.46	0.90	0.90	1.31	0.20							
Clostridium_sensu_stricto_1	7	0.36	0.20	0.39	0.23	0.15	0.16	0.33	0.35	0.48	<0.0001	0.67	0.67	0.97	0.4
	14	0.59	0.17	0.10	0.05	0.03	0.21	0.35							
	21	0.24	0.18	0.38	0.26	0.29	0.18	0.33							

	42	2.71	2.99	3.05	3.02	1.99	3.54	0.33							
Clostridium_sensu_stri	7	0.01	0.00	0.00	0.02	0.01	0.00	0.02	0.48	0.47	0.0001	0.24	0.05	0.48	0.29
cto_6	14	0.03	0.03	0.01	0.00	0.00	0.01	0.02							
	21	0.03	0.00	0.11	0.02	0.02	0.03	0.02							
	42	0.06	0.06	0.06	0.01	0.13	0.08	0.02							
Colidextribacter	7	0.32	0.50	0.10	0.06	0.12	0.04	0.07	0.38	0.23	0.0001	0.44	0.15	0	0.8
	14	0.04	0.11	0.01	0.02	0.06	0.07	0.08							
	21	0.04	0.05	0.03	0.06	0.06	0.12	0.07							
	42	0.05	0.05	0.03	0.05	0.03	0.03	0.07							
Collinsella	7	0.31	0.50	0.08	0.15	0.14	0.06	0.08	0.18	0.16	0.01	0.29	0.4	0.18	0.71
	14	0.02	-0.08	0.03	0.01	0.00	0.03	0.09							
	21	0.22	0.13	0.07	0.10	0.13	0.12	0.08							
	42	0.09	0.04	0.02	0.05	0.07	0.08	0.08							
Coprococcus	7	1.25	1.12	0.95	0.73	0.86	0.38	0.22	0.85	0.03	<0.0001	0.7	0.62	0.1	0.45
	14	0.91	1.00	1.09	1.00	0.89	1.12	0.24							
	21	2.15	1.66	1.63	1.20	1.47	1.44	0.22							
	42	1.54	1.63	1.54	1.63	1.28	1.79	0.22							
Denitrobacterium	7	0.06	0.03	0.00	0.02	0.05	0.02	0.01	0.77	0.12	0.02	0.22	0.19	0.83	0.67
	14	0.02	0.00	0.04	0.00	0.00	0.01	0.02							
	21	0.04	0.04	0.03	0.03	0.03	0.03	0.01							
	42	0.03	0.02	0.03	0.01	0.02	0.02	0.01							
Desulfovibrio	7	0.75	1.04	0.86	0.80	0.52	0.69	0.14	0.13	0.001	<0.0001	0.11	0.22	0.04	0.77
	14	0.63	0.92	0.93	0.34	0.27	0.26	0.15							
	21	0.39	0.55	0.24	0.24	0.18	0.21	0.14							
	42	0.61	1.17	0.62	0.54	0.91	0.69	0.14							
dgA_11_gut_group	7	0.62	0.56	0.53	0.47	0.39	0.90	0.20	0.3	0.55	<0.0001	0.36	0.46	0.78	0.77

	14	0.18	0.15	0.19	0.16	0.05	0.34	0.21						
	21	0.05	0.07	0.10	0.06	0.02	0.10	0.20						
	42	0.50	0.65	0.60	0.31	1.07	0.97	0.20						
Dorea	7	0.05	0.00	0.07	0.05	0.03	0.08	0.10	0.47	0.15	<0.0001	0.18	0.97	0.62	0.75
	14	0.52	0.32	0.40	0.38	0.32	0.27	0.11						
	21	1.00	0.80	0.74	0.59	0.76	0.79	0.10						
	42	0.33	0.20	0.24	0.22	0.23	0.27	0.10						
Escherichia_Shigella	7	0.94	0.50	0.37	0.68	0.28	0.65	0.19	0.24	0.26	<0.0001	0.66	0.42	0.03	0.33
	14	0.43	0.25	0.10	0.71	0.72	0.65	0.20						
	21	0.04	0.07	0.23	0.63	0.13	0.11	0.19						
	42	0.07	0.39	0.02	0.01	0.03	-0.01	0.19						
Faecalibacterium	7	1.40	0.69	1.15	1.08	1.09	1.07	0.52	0.92	0.29	<0.0001	0.07	0.76	0.13	0.62
	14	1.83	1.49	1.84	2.44	3.46	2.33	0.55						
	21	4.28	3.20	3.29	3.66	4.42	3.65	0.52						
	42	3.37	1.77	2.42	1.62	2.68	2.88	0.52						
Family_XIII_AD3011_group	7	0.16	0.13	0.24	0.20	0.34	0.34	0.04	0.54	0.14	<0.0001	0.87	0.51	0.07	0.62
	14	0.03	0.01	0.00	0.01	0.01	0.02	0.05						
	21	0.00	0.04	0.03	0.01	0.01	0.05	0.04						
	42	0.05	0.07	0.04	0.08	0.04	0.03	0.04						
Family_XIII_UCG_001	7	0.01	0.02	0.02	0.00	0.02	0.00	0.02	0.46	0.51	<0.0001	0.56	0.4	0.11	0.71
	14	0.02	0.01	0.01	0.04	0.05	0.02	0.02						
	21	0.05	0.07	0.05	0.02	0.07	0.06	0.02						
	42	0.03	0.00	0.02	0.01	0.02	0.04	0.02						
Fibrobacter	7	0.05	0.14	0.12	0.22	0.10	0.05	0.06	0.94	0.73	0.16	0.59	0.98	0.71	0.22
	14	0.06	0.10	0.05	0.08	0.04	0.11	0.06						
	21	0.10	0.12	0.02	0.01	0.01	0.06	0.06						

	42	0.16	0.09	0.13	0.08	0.13	0.15	0.06						
Fournierella	7	0.41	0.16	0.48	0.56	0.33	0.53	0.11	0.4	0.14	<0.0001	0.72	0.41	0.79	1
	14	0.01	0.05	0.05	0.08	0.07	0.12	0.12							
	21	0.08	0.05	0.18	0.14	0.21	0.24	0.11							
	42	0.24	0.28	0.30	0.26	0.35	0.19	0.11							
Frisingicoccus	7	0.12	0.19	0.43	0.44	0.20	0.34	0.09	0.42	0.83	<0.0001	0.18	0.88	0.63	0.91
	14	0.07	0.09	0.18	0.24	0.09	0.10	0.10							
	21	0.01	0.00	0.07	0.03	0.00	-0.01	0.09							
	42	0.00	0.03	0.14	0.00	0.00	-0.01	0.09							
Fusicatenibacter	7	0.04	0.00	0.06	0.09	0.19	0.05	0.07	0.09	0.03	<0.0001	0.59	0.02	0.69	0.12
	14	0.06	0.08	0.11	0.20	0.24	0.15	0.08							
	21	0.34	0.28	0.40	0.33	0.25	0.68	0.07							
	42	0.35	0.21	0.44	0.31	0.36	0.39	0.07							
Holdemanella	7	0.47	0.73	0.45	0.54	0.46	0.10	0.13	0.7	0.33	<0.0001	0.88	0.25	0.42	0.56
	14	0.19	0.22	0.22	0.11	0.13	0.08	0.13							
	21	0.47	0.23	0.33	0.38	0.34	0.48	0.13							
	42	0.25	0.19	0.29	0.13	0.35	0.22	0.13							
Horsej_a03	7	0.01	0.07	0.01	0.00	0.00	0.00	0.03	0.46	0.84	<0.0001	0.5	0.62	0.03	0.65
	14	0.00	0.00	0.00	0.00	0.01	0.00	0.03							
	21	0.03	0.04	0.05	0.00	0.00	0.01	0.03							
	42	0.06	0.08	0.06	0.18	0.13	0.06	0.03							
Incertae_Sedis	7	0.05	0.04	0.02	0.23	0.12	0.07	0.04	0.19	0.36	<0.0001	0.82	0.68	0	0.02
	14	0.14	0.13	0.07	0.12	0.05	0.12	0.04							
	21	0.29	0.11	0.18	0.11	0.17	0.13	0.04							
	42	0.21	0.22	0.18	0.24	0.23	0.27	0.04							
Intestinibacter	7	0.04	0.05	0.15	0.10	0.16	0.13	0.09	1	0.62	<0.0001	0.57	0.98	0.85	0.8

	14	0.21	0.24	0.15	0.28	0.20	0.28	0.10						
	21	0.88	0.86	0.80	0.78	0.84	0.85	0.09						
	42	0.34	0.26	0.15	0.21	0.23	0.31	0.09						
Intestinimonas	7	0.01	0.12	0.16	0.11	0.07	0.00	0.04	0.99	0.47	<0.0001	0.58	0.86	0.72	0.09
	14	0.06	0.02	0.04	0.04	0.01	0.03	0.04						
	21	0.20	0.15	0.18	0.20	0.15	0.18	0.04						
	42	0.13	0.07	0.10	0.10	0.13	0.12	0.04						
Lachnoclostridium	7	0.00	0.02	0.04	0.05	0.07	0.03	0.05	0.68	0.21	<0.0001	0.55	0.97	0.06	0.43
	14	0.14	0.02	0.09	0.16	0.26	0.16	0.05						
	21	0.03	0.00	0.00	0.00	0.00	0.00	0.05						
	42	0.03	0.10	0.02	0.03	0.03	0.00	0.05						
Lachnospira	7	0.51	0.34	0.49	0.38	0.54	0.45	0.17	0.66	0.32	<0.0001	0.42	0.4	0.39	0.9
	14	0.96	0.48	0.90	0.84	0.82	0.86	0.18						
	21	1.04	1.09	0.97	0.82	0.82	0.70	0.17						
	42	0.64	0.82	1.14	0.62	0.81	0.82	0.17						
Lachnospiraceae_AC2	7	1.13	1.72	1.48	1.16	0.99	0.93	0.43	0.4	0.8	0.12	0.83	0.73	0.18	0.6
044_group	14	1.82	0.69	0.67	0.76	0.56	0.67	0.46						
	21	0.74	0.57	0.50	0.91	0.42	0.74	0.43						
	42	0.51	1.10	0.75	1.77	1.51	0.73	0.43						
Lachnospiraceae_FCS	7	0.09	0.08	0.15	0.02	0.06	0.00	0.04	0.46	0.004	0.16	0.22	0.9	0.43	0.87
020_group	14	0.08	0.01	0.04	0.03	0.03	-0.01	0.04						
	21	0.13	0.13	0.11	0.08	0.06	0.04	0.04						
	42	0.06	0.03	0.03	0.05	0.05	0.00	0.04						
Lachnospiraceae_NC2	7	0.09	0.29	0.12	0.04	0.04	0.09	0.05	0.73	0.002	0.003	0.35	0.5	0.34	0.06
004_group	14	0.08	0.06	0.00	0.00	0.01	0.00	0.05						
	21	0.18	0.09	0.11	0.01	0.00	0.01	0.05						

	42	0.07	0.22	0.16	0.11	0.01	0.02	0.05							
Lachnospiraceae_ND3	7	0.00	0.00	0.05	0.03	0.02	0.06	0.05	0.65	0.39	<0.0001	0.38	0.1	0.3	0.71
007_group	14	0.06	0.07	0.03	0.12	0.05	0.06	0.06							
	21	0.35	0.30	0.12	0.17	0.24	0.13	0.05							
	42	0.14	0.13	0.11	0.05	0.06	0.16	0.05							
Lachnospiraceae_NK3	7	0.00	0.00	0.01	0.00	0.00	0.00	0.22	0.66	0.02	<0.0001	0.89	0.91	0.01	0.97
A20_group	14	0.08	0.22	0.32	0.23	0.00	0.04	0.24							
	21	1.56	1.37	1.59	0.78	0.51	1.03	0.22							
	42	0.05	0.05	0.01	0.03	0.02	0.01	0.22							
Lachnospiraceae_NK4	7	2.13	2.19	2.39	1.86	1.81	1.27	0.43	0.68	0.003	<0.0001	0.16	0.73	0.13	0.97
A136_group	14	2.79	2.80	3.24	2.42	1.52	2.28	0.46							
	21	1.60	2.32	2.66	1.18	1.42	1.37	0.43							
	42	0.67	1.22	1.14	1.49	0.90	1.14	0.43							
Lachnospiraceae_NK4	7	0.01	0.02	0.05	0.06	0.03	0.06	0.06	0.75	0.52	<0.0001	0.54	0.79	0.02	0.31
B4_group	14	0.09	0.03	0.18	0.12	0.25	0.18	0.07							
	21	0.10	0.07	0.10	0.16	0.01	0.06	0.06							
	42	0.20	0.38	0.27	0.17	0.09	0.15	0.06							
Lachnospiraceae_UCG	7	0.21	0.03	0.04	0.11	0.17	0.38	0.09	0.68	0.01	<0.0001	0.44	0.13	0.78	0.18
_001	14	0.11	0.21	0.35	0.40	0.47	0.34	0.10							
	21	0.82	0.62	0.45	0.73	0.77	0.60	0.09							
	42	0.09	0.06	0.09	0.20	0.25	0.08	0.09							
Lachnospiraceae_UCG	7	0.03	0.00	0.04	0.16	0.07	0.04	0.06	0.24	0.12	0.56	0.83	0.42	0.53	0.82
_003	14	0.00	0.05	0.09	0.08	0.04	0.22	0.06							
	21	0.04	0.01	0.13	0.03	0.02	0.08	0.06							
	42	0.01	0.00	0.01	0.06	0.04	0.06	0.06							
	7	0.01	0.00	0.00	0.00	0.00	0.07	0.04	0.13	6E-04	0.0004	0.02	0.1	<0.0001	0.05

Lachnospiraceae_UCG	14	0.01	0.00	0.01	0.07	0.35	0.13	0.04						
_004	21	0.09	0.04	0.03	0.08	0.19	0.18	0.04						
	42	0.10	0.09	0.10	0.03	0.06	0.05	0.04						
Lachnospiraceae_XPB	7	0.35	0.32	0.32	0.23	0.36	0.42	0.08	0.83	0.05	<0.0001	0.29	0.62	0.01	0.54
1014_group	14	0.42	0.43	0.14	0.09	0.03	0.09	0.08							
	21	0.01	0.01	0.00	0.01	0.00	0.00	0.08							
	42	0.00	0.06	0.03	0.00	0.00	0.00	0.08							
Lactobacillus	7	6.07	10.0	9.77	11.3	11.5	11.2	1.66	0.91	0.62	<0.0001	0.96	0.71	0.21	0.53
	14	12.0	11.5	12.0	10.9	10.5	10.0	1.79							
	21	14.6	14.4	15.7	15.2	14.8	14.0	1.66							
	42	7.28	4.47	2.25	4.58	5.47	4.88	1.66							
Mailhella	7	0.11	0.04	0.09	0.05	0.03	0.05	0.02	0.97	0.17	<0.0001	0.83	0.28	0.33	0.79
	14	0.01	0.02	0.00	0.01	0.01	0.02	0.02							
	21	0.00	0.00	0.00	0.00	0.00	0.00	0.02							
	42	0.03	0.07	0.00	0.01	0.02	0.01	0.02							
Marvinbryantia	7	0.17	0.12	0.13	0.20	0.13	0.07	0.06	0.73	0.87	<0.0001	0.92	0.22	0.54	0.94
	14	0.11	0.16	0.08	0.07	0.12	0.09	0.06							
	21	0.31	0.32	0.37	0.28	0.26	0.34	0.06							
	42	0.26	0.22	0.30	0.27	0.26	0.40	0.06							
Megasphaera	7	0.80	0.28	1.24	0.61	0.91	0.17	0.38	0.91	0.61	<0.0001	0.79	0.73	0.001	0.27
	14	0.65	1.47	1.22	0.61	0.73	0.62	0.40							
	21	2.08	1.87	2.18	3.40	2.76	3.37	0.38							
	42	1.01	0.60	0.61	0.58	0.68	0.75	0.38							
Methanosphaera	7	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.21	0.32	<0.0001	0.15	0.17	0.17	0.35
	14	0.03	0.01	0.03	0.03	0.00	0.05	0.08							
	21	0.32	0.30	0.11	0.20	0.04	0.04	0.08							

	42	0.74	0.51	0.54	0.57	0.49	0.83	0.08							
Mitsuokella	7	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.97	0.28	0.001	0.41	0.26	0.15	0.18
	14	0.00	0.01	0.01	0.04	0.00	0.03	0.02							
	21	0.04	0.03	0.01	0.03	0.10	0.05	0.02							
	42	0.05	0.00	0.05	0.01	0.03	0.02	0.02							
Monoglobus	7	0.04	0.03	0.12	0.12	0.10	0.11	0.03	0.76	0.13	0.004	0.62	0.49	0.52	0.77
	14	0.03	0.03	0.01	0.04	0.02	0.03	0.03							
	21	0.00	0.06	0.03	0.05	0.06	0.02	0.03							
	42	0.00	0.00	0.03	0.03	0.03	0.07	0.03							
NK4A214_group	7	0.80	0.99	0.93	1.20	0.42	0.84	0.16	0.39	0.04	<0.0001	0.25	0.41	0.8	0.28
	14	0.60	0.50	0.44	0.32	0.19	0.40	0.18							
	21	0.70	0.47	0.58	0.44	0.28	0.32	0.16							
	42	0.47	0.76	0.78	0.48	0.48	0.73	0.16							
Not_Assigned	7	19.8	22.6	16.2	17.9	14.6	15.9	1.27	0.56	0.01	<0.0001	0.04	0.43	0.02	0.17
	14	15.9	15.1	14.4	12.4	13.5	15.5	1.36							
	21	12.6	13.1	11.9	10.8	9.7	10.3	1.27							
	42	12.8	16.1	14.8	16.5	15.5	15.5	1.27							
Olsenella	7	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.95	0.09	<0.0001	0.8	0.49	0.39	0.27
	14	0.00	0.00	0.01	0.01	0.00	0.00	0.02							
	21	0.03	0.07	0.07	0.04	0.05	0.01	0.02							
	42	0.07	0.03	0.03	0.02	0.01	0.04	0.02							
Oribacterium	7	0.09	0.00	0.18	0.18	0.21	0.16	0.09	0.41	0.06	<0.0001	0.63	0.19	0.01	0.23
	14	0.07	0.28	0.21	0.19	0.40	0.26	0.10							
	21	0.38	0.29	0.26	0.38	0.29	0.26	0.09							
	42	0.40	0.45	0.36	0.07	0.26	0.51	0.09							
Oscillibacter	7	0.45	0.49	1.27	0.86	0.83	0.88	0.17	0.66	0.47	<0.0001	0.6	0.29	0.54	0.4

	14	0.61	0.52	0.36	0.36	0.34	0.31	0.18						
	21	0.23	0.32	0.22	0.18	0.09	0.14	0.17						
	42	0.12	0.32	0.20	0.18	0.15	0.18	0.17						
Oscillospira	7	0.25	0.32	0.19	0.38	0.42	0.54	0.07	0.42	0.27	<0.0001	0.26	0.55	0.02	0.35
	14	0.26	0.36	0.09	0.18	0.15	0.15	0.08						
	21	0.08	0.12	0.11	0.10	0.13	0.10	0.07						
	42	0.12	0.09	0.20	0.05	0.17	0.15	0.07						
Parabacteroides	7	0.42	0.73	0.38	0.04	0.23	0.20	0.14	0.44	0.17	0.004	0.65	0.87	0.09	0.58
	14	0.20	0.37	0.04	0.02	0.09	0.12	0.15						
	21	0.02	0.11	0.15	0.15	0.10	0.05	0.14						
	42	0.19	0.29	0.10	0.12	0.22	0.21	0.14						
Peptococcus	7	0.13	0.12	0.13	0.14	0.19	0.07	0.04	0.48	0.05	<0.0001	0.36	0.42	0	0.91
	14	0.03	0.06	0.08	0.05	0.02	0.04	0.04						
	21	0.23	0.30	0.22	0.12	0.16	0.06	0.04						
	42	0.07	0.06	0.10	0.11	0.09	0.08	0.04						
Phascolarctobacterium	7	2.03	2.00	2.23	1.79	1.44	1.84	0.27	0.85	0.04	<0.0001	0.33	0.06	0.18	0.13
	14	2.72	2.07	1.14	1.15	1.74	1.36	0.28						
	21	1.20	1.25	1.28	1.16	1.27	1.50	0.27						
	42	0.85	1.28	1.23	0.89	1.20	1.06	0.27						
possible_genus_Sk018	7	0.00	0.04	0.03	0.07	0.06	0.04	0.06	0.22	0.12	0.001	0.21	0.14	0.002	0.23
	14	0.02	0.00	0.02	0.02	0.00	0.05	0.06						
	21	0.00	0.00	0.00	0.00	0.00	0.00	0.06						
	42	0.00	0.33	0.38	0.02	0.06	0.01	0.06						
Prevotella	7	22.0	18.5	20.1	20.1	19.6	20.1	2.30	0.8	0.11	<0.0001	0.41	0.45	0.31	0.03
	14	17.5	26.7	29.8	30.2	30.4	26.0	2.46						
	21	18.0	21.5	19.5	22.5	21.7	22.5	2.30						

	42	16.5	11.6	14.5	13.7	15.5	14.4	2.30							
Prevotellaceae_NK3B31_group	7	1.88	3.23	4.16	3.10	5.02	5.85	0.61	0.91	0.92	<0.0001	0.41	<0.0001	0.003	0.04
	14	6.98	4.55	2.44	3.74	3.55	3.75	0.61							
	21	1.42	1.82	1.25	1.37	1.16	1.16	0.61							
	42	3.90	3.52	3.83	4.39	3.13	3.11	0.61							
Prevotellaceae_UCG_001	7	0.53	0.73	0.69	0.64	0.52	0.70	0.14	0.85	0.92	<0.0001	0.84	0.54	0.98	0.86
	14	0.44	0.05	0.20	0.25	0.07	0.25	0.15							
	21	-0.01	0.02	-0.01	0.04	0.00	0.03	0.14							
	42	0.01	0.20	0.14	0.13	0.14	0.12	0.14							
Prevotellaceae_UCG_003	7	0.69	1.09	2.28	1.63	1.92	2.07	0.23	0.41	0.49	<0.0001	0.73	0.004	0.05	0.05
	14	1.38	1.23	0.72	0.70	1.14	1.07	0.25							
	21	0.31	0.36	0.34	0.31	0.45	0.44	0.23							
	42	1.21	1.15	1.23	1.41	0.86	0.81	0.23							
Prevotellaceae_UCG_004	7	0.32	0.32	0.31	0.21	0.18	0.11	0.06	0.96	0.33	<0.0001	0.98	0.93	0.04	0.79
	14	0.03	0.02	-0.01	0.03	0.00	0.12	0.06							
	21	0.00	0.00	0.01	0.00	0.00	0.00	0.06							
	42	0.02	0.02	0.03	0.03	0.11	0.01	0.06							
Pseudobutyrivibrio	7	0.00	0.00	0.05	0.00	0.02	0.02	0.06	0.35	0.98	<0.0001	0.76	0.9	0.92	0.27
	14	0.02	0.01	0.08	0.03	0.02	0.04	0.06							
	21	0.19	0.20	0.14	0.11	0.06	0.31	0.06							
	42	0.00	0.05	0.05	0.04	0.08	0.05	0.06							
Rikenellaceae_RC9_gut_group	7	4.03	4.48	3.42	4.20	2.66	3.16	0.58	0.55	0.06	<0.0001	0.24	0.29	0.69	0.95
	14	3.03	4.42	3.56	2.55	2.63	2.93	0.62							
	21	1.84	2.38	2.17	2.08	1.85	1.65	0.58							
	42	3.35	4.89	4.59	3.63	4.37	3.82	0.58							
Roseburia	7	2.10	1.39	1.75	2.11	1.84	1.52	0.41	0.4	0.03	0.24	0.78	0.71	0.21	0.44

	14	2.92	2.02	2.02	1.82	1.45	1.53	0.44						
	21	2.01	2.01	1.67	1.28	1.21	0.94	0.41						
	42	1.54	2.30	2.37	1.94	1.71	1.07	0.41						
Ruminococcus	7	1.78	1.38	2.19	1.87	1.33	1.32	0.30	0.62	0.81	0.04	0.4	0.17	0.38	0.42
	14	1.89	2.40	1.57	1.43	1.73	1.74	0.33						
	21	2.17	2.00	2.19	2.21	2.39	1.79	0.30						
	42	1.32	2.13	1.82	1.26	1.52	2.01	0.30						
Selenomonas	7	0.71	0.12	0.14	0.07	0.22	-0.01	0.17	0.73	0.11	<0.0001	0.72	0.46	0.2	0.25
	14	0.37	0.40	0.62	0.34	0.07	0.21	0.18						
	21	0.75	0.82	0.79	0.56	0.54	0.72	0.17						
	42	0.37	0.26	0.47	0.32	0.53	0.62	0.17						
Shuttleworthia	7	0.01	0.06	0.02	0.05	0.04	0.02	0.03	0.71	0.19	0.02	0.57	0.69	0.26	0.49
	14	0.03	0.06	0.07	0.09	0.11	0.12	0.03						
	21	0.04	0.03	0.06	0.07	0.04	0.06	0.03						
	42	0.02	0.01	0.09	0.03	0.07	0.02	0.03						
Solobacterium	7	0.08	0.13	0.30	0.22	0.20	0.18	0.08	0.46	0.04	<0.0001	0.65	0.27	0.02	0.44
	14	0.15	0.23	0.17	0.15	0.18	0.16	0.08						
	21	0.71	0.52	0.58	0.33	0.44	0.34	0.08						
	42	0.48	0.47	0.64	0.34	0.41	0.54	0.08						
Sphaerochaeta	7	0.02	0.23	0.04	0.11	0.06	0.06	0.08	0.84	0.95	<0.0001	0.23	0.97	0.65	0.59
	14	0.01	0.05	0.00	0.03	0.00	0.02	0.08						
	21	0.03	0.06	0.04	0.00	0.00	0.01	0.08						
	42	0.14	0.28	0.32	0.40	0.25	0.25	0.08						
Streptococcus	7	0.81	0.93	0.78	0.00	0.05	-0.05	0.51	0.94	0.52	0.002	0.96	1	0	1
	14	0.00	0.03	0.01	0.06	0.03	0.14	0.55						
	21	0.25	0.01	0.07	1.71	1.77	1.85	0.51						

	42	1.25	1.41	0.92	0.83	1.03	0.76	0.51							
Subdoligranulum	7	0.23	0.37	0.81	0.34	0.69	0.32	0.17	0.69	0.42	<0.0001	0.29	0.4	0.66	0.37
	14	0.47	0.47	0.72	0.42	0.55	0.51	0.18							
	21	1.40	1.05	1.21	1.28	1.23	1.28	0.17							
	42	1.44	1.32	1.23	1.09	1.12	1.17	0.17							
Succinivibrio	7	3.23	1.46	1.46	0.78	4.23	1.93	0.61	0.6	0.25	0.005	0.12	0.71	0.46	0.03
	14	0.88	1.03	1.30	2.19	1.91	2.06	0.65							
	21	1.03	0.74	1.15	0.73	1.15	1.12	0.61							
	42	1.09	1.23	1.02	1.39	1.25	0.45	0.61							
Sutterella	7	0.00	0.00	0.03	0.00	0.00	0.03	0.04	0.49	0.38	<0.0001	0.91	0.87	0.36	0.65
	14	0.00	0.01	0.01	0.02	0.00	0.07	0.04							
	21	0.03	0.10	0.06	0.01	0.02	0.01	0.04							
	42	0.18	0.08	0.16	0.10	0.13	0.11	0.04							
Terrisporobacter	7	0.10	0.13	0.18	0.13	0.08	0.04	0.15	0.52	0.9	<0.0001	0.71	0.76	0.8	0.64
	14	0.10	0.07	0.05	0.04	-0.01	0.03	0.16							
	21	0.16	0.09	0.21	0.11	0.16	0.12	0.15							
	42	1.29	1.45	1.55	1.62	1.20	1.76	0.15							
Treponema	7	3.80	3.13	3.38	3.04	1.94	3.04	0.77	0.58	0.13	<0.0001	0.41	0.92	0.57	0.23
	14	3.11	2.20	2.23	1.50	0.84	1.67	0.82							
	21	0.75	0.57	0.94	0.27	0.19	0.18	0.77							
	42	3.23	5.72	4.77	5.33	3.17	5.27	0.77							
Turicibacter	7	0.08	0.04	0.03	0.02	0.03	0.00	0.03	0.48	0.39	<0.0001	0.67	0.3	0.22	0.88
	14	0.06	0.06	0.00	0.00	0.01	0.00	0.03							
	21	0.01	0.02	0.00	0.00	0.01	0.00	0.03							
	42	0.10	0.06	0.10	0.16	0.04	0.15	0.03							
UCG_002	7	1.83	1.57	1.10	1.40	0.95	1.13	0.22	0.89	0.22	<0.0001	0.43	0.1	0.14	0.98

	14	0.66	0.52	0.57	0.58	0.17	0.35	0.24						
	21	0.51	0.70	0.68	0.35	0.37	0.59	0.22						
	42	0.66	1.03	1.03	0.86	1.13	1.44	0.22						
UCG_003	7	0.13	0.13	0.04	0.15	0.04	0.05	0.03	0.22	0.17	<0.0001	0.2	0.1	0.86	0.62
	14	0.00	0.10	0.01	0.00	0.00	0.02	0.03						
	21	0.04	0.01	0.01	0.01	0.00	0.00	0.03						
	42	0.00	0.00	0.00	0.00	0.00	0.00	0.03						
UCG_004	7	0.24	0.10	0.10	0.55	0.03	0.03	0.14	0.35	0.43	<0.0001	0.29	0.42	0.7	0.84
	14	0.10	0.10	0.06	0.08	0.04	0.07	0.15						
	21	0.18	0.15	0.24	0.05	0.10	0.10	0.14						
	42	0.47	0.67	0.65	0.62	0.47	0.42	0.14						
UCG_005	7	0.22	1.04	1.00	0.92	0.65	0.69	0.24	0.53	0.15	<0.0001	0.14	0.73	0.76	0.05
	14	0.97	0.83	0.59	0.51	0.60	0.48	0.26						
	21	0.80	0.66	0.46	0.40	0.44	0.43	0.24						
	42	1.34	2.42	1.60	1.82	1.37	1.63	0.24						
UCG_008	7	0.06	0.06	0.09	0.44	0.32	0.32	0.26	0.05	<0.0001	<0.0001	0.99	0.07	0.001	0.98
	14	0.04	0.05	0.08	0.65	0.45	0.78	0.28						
	21	0.18	0.24	0.87	1.55	1.92	2.10	0.26						
	42	3.98	3.12	3.84	4.99	4.11	4.94	0.26						

Treatments were (1) milk replacer, commercial milk substitute rich in animal fat and coconut oil (CO), milk substitute rich in polar lipids (PO), or milk substitute rich in soy lipids (SO) until day 7; and (2) diet, solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD) from d 7 to d 21. From d 21 to 42 all piglets received a commercial diet.

Supplemental Table 3. Effects of milk replacers (MR) and diets on alpha diversity methods at feature level in piglets on the nursery phase.

		PD			SD				*P*-value							
Method	Time	CO	PO	SO	CO	PO	SO	SEM	MR	Diet	Time	MR*Diet	Time*MR	Time*Diet	MR*Diet*Time	
Chao1	7	247	266	255	270	267	261	16.9	0.93	0.26	<0.0001	0.76	0.55	0.05	0.94	
	14	267	259	255	262	248	254	18.2								
	21	285	277	279	251	231	239	16.8								
	42	286	319	322	299	322	292	16.6								
Shannon	7	4.74	4.70	4.82	4.83	4.74	4.79	0.09	0.99	0.35	<0.0001	0.99	0.09	0.14	0.23	
	14	4.89	4.81	4.70	4.72	4.79	4.82	0.09								
	21	4.85	4.76	4.73	4.66	4.61	4.64	0.08								
	42	4.91	5.13	5.12	4.97	5.09	4.99	0.07								
Simpson	7	0.98	0.98	0.99	0.99	0.98	0.98	0.004	0.84	0.76	<0.0001	0.54	0.62	0.81	0.27	
	14	0.99	0.98	0.98	0.98	0.99	0.99	0.003								
	21	0.98	0.98	0.98	0.98	0.98	0.98	0.002								
	42	0.99	0.99	0.99	0.99	0.99	0.99	0.002								

Treatments were: 1) Milk Replacer: commercial milk substitute rich in animal fat and coconut oil (CO); milk substitute rich in polar lipids (PO) or milk substitute rich in soy lipids (SO) until day 7; 2) Diet: solid feed containing soy lipids (SD) or lipids from cow milk fat globular membranes (PD) from d 7 to d 21. From d 21 to 42 all piglets received a commercial diet.

CONCLUSION

This thesis explored the impact of nutritional modulation in pigs using omics approaches, revealing how dietary interventions can shape metabolism, lipidomic and microbial profiles to improve health and productivity.

In lactating sows, TZD supplementation increased the expression of genes related to lipid and protein synthesis in the mammary gland, resulting in reduced SFA and increased MUFA in milk.

For weaned piglets, diets enriched with polar lipids significantly modified the plasma lipidomic profile, increasing the levels of sphingolipids and phospholipids, while improving beneficial genera of the gut microbiota such as *Coprococcus* and *Roseburia*. Conversely, the soybean-based diet increased pro-inflammatory lipid mediators, suggesting a diet-dependent effect on inflammation. Although no immediate inflammatory markers were altered, these dietary interventions promoted beneficial changes in microbiota and lipid profiles, indicating potential long-term benefits for immune regulation and metabolism.

The findings highlight the value of omics-based nutritional strategies in refining feed formulations and improving animal health. Future studies should evaluate these interventions under more challenging weaning conditions to further explore their impacts on growth, immunity, and welfare in swine production.