A Multi-omics Approach to Understanding the Effects of Common Feeding Strategies on Diet-Microbe-Host Interaction in Weaned Pigs

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Animal Science

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Abstract

Post-weaning diarrhea (PWD) is a serious challenge in global swine production systems with significant impacts on antimicrobial use and production economics. The outcomes of this multi-factorial disease are influenced by many nutritional and management factors which affect the colonization and expansion of gastrointestinal pathogens. To understand factors that contribute to the effectiveness of common nutritional strategies, more information is needed on how they impact functional characteristics of the gut microbiota as well as host response. In order to elucidate the effects of three such strategies on diet-microbiota-host interactions, benzoic acid (BA) and enzymes, an additive blend with medium-chain fatty acids (MCFAs), and protein to fibre ratios were each examined in one of three studies conducted.

To examine the effects of BA, dietary enzymes, or a combination of both on gut microbiota and metabolome, pigs were assigned to one of four diets 7 days after weaning: a control diet or a diet with the addition of benzoic acid, dietary enzymes, or both and fed ad libitum for 21 to 22 days. Decreased diarrhea incidence from experimental day 8-14 was observed in pigs fed both BA and enzymes. Benzoic acid altered cecal microbiota composition and decreased α-diversity, while dietary enzymes increased fibre-fermenting bacterial taxa. Metabolite comparisons were limited by compound identification constraints, however seventeen cecal metabolites differed among the diets demonstrating the effects of benzoic acid and enzymes on gut microbiota and microbial metabolites.

In the second study described, the effects of a dietary additive blend containing MCFAs, target-release butyrate, organic acids, and a phenolic compound (MCOA) was evaluated. Changes in microbiota and metabolome were compared between the MCOA and control diets at Day 3, 5, 7 and 14 post-weaning. Many pronounced changes in metabolome were identified in MCOA-fed

animals 7 days post-weaning, including improved whole-body metabolism, alterations in bile-associated metabolites, and increases in beneficial tryptophan metabolites. These changes in metabolome were identified alongside a tendency toward increased *Lactobacillus* sp. in the small intestine, and improved indicators of microbial succession in the colonic microbiota. Taken together, these results indicate that MCOA may help improve host metabolism and aid in microbiota succession through support of bile acid production and secretion post-weaning.

To assess the mechanisms by which dietary protein and fibre may contribute to PWD outcomes, the final study described was conducted using highly controlled, semi-purified diets. Results of this work identified many critical alterations in host metabolome and transcriptome in response to a standard (low protein) post-weaning diet. While pathogen proliferation is cited as a common concern with respect to feeding high protein diets, this study showed subtle alterations to the cecal microbiota with no indication of pathobiont expansion. No alterations in the ileal microbiota were observed. This study challenges current industry practice, demonstrating a detrimental effect of decreasing dietary protein on key intestinal cell and muscle accretion pathways. The lack of substantial change in gut microbiota composition suggests that with carefully chosen ingredients, higher protein diets may better support the needs of weaned pigs.

The metabolite and microbiota changes described through these multi-omic techniques provide a deeper understanding of the impacts of these three strategies on gut microbiota, metabolism, and host response. Through focusing on functional characteristics indicative of recovery from post-weaning stress, these studies further provide important insight into alterations in holobiont metabolic networks that may underly the mechanism of action for these dietary strategies.

Preface

This thesis is an original work by Natalie Diether.

Chapter 2 has been published as Diether, N. E. and Willing, B. P. Microbial fermentation of dietary protein: An important factor in diet-microbe-host interaction. Microorganisms. 2019;7:19. NED wrote the review, created figures, and compiled references. BPW supervised, edited, and approved the review.

Chapter 3 has been published as Diether, N. E., Nam, S., Fouhse, J., le Thanh, B. V., Stothard, P., Zijlstra, R. T., Harynuk, J., de la Mata, P., Willing, B. P. Dietary benzoic acid and supplemental enzymes alter fiber-fermenting taxa and metabolites in the cecum of weaned pigs. 2022;100:skac324. NED performed data analysis and wrote the manuscript. SN conducted GCxGC-TOFMS, and assisted with data analysis and writing the manuscript. JF assisted with data collection and analysis. BVT assisted with data collection and analysis. P. S. supervised data analysis and visualization. RTZ designed the experiment, supervised animal work and performance data analysis. JH supervised GCxGC-TOFMS and analysis. PM performed GCxGC-TOFMS, supervised data analysis and assisted with writing the manuscript. BPW supervised microbiota sequencing, data analysis, and visualization.

Chapter 4 has been submitted to PLoS One as Diether, N. E., Hulshof, T. G., Willing, B. P., van Kempen, T. G. A blend of medium-chain fatty acids, butyrate, organic acids, and a phenolic compound accelerates microbial maturation in newly weaned piglets. NED analyzed the microbiota and metabolomics data and drafted the manuscript. TGH conceptualized and designed the experiment, analyzed performance data, and reviewed and edited the manuscript. BPW

oversaw analysis and data integration, reviewed, and edited the manuscript. TGK conceptualized and designed the experiment, reviewed, and edited the manuscript.

The study in Chapter 5 was designed by NED, JMF, and BPW. RTZ and MG assisted with diet formulation. NED conducted the experiment, collected and analyzed the data. PS and BPW supervised data analysis and integration and edited the chapter.

Dedication

For Helen: discovering the world with you reinvigorates my love for science every day. You are my greatest adventure.

"All zoology is really ecology. We cannot fully understand the lives of animals without understanding our microbes and our symbioses with them."

- Ed Yong, I Contain Multitudes

Acknowledgements

We often think of good luck as things that happen, but the best kind of luck comes in the form of wonderful people. I have had the honour of working with and getting to know many wonderful people during my PhD. I am grateful for the supervision of Dr. Paul Stothard and Dr. Ben Willing. Thank-you both for your mentorship, for letting me pursue my own ideas, and supporting me to take on collaborations and responsibilities in teaching and governance. This PhD was sprinkled with a lot of life events, and your unwavering understanding and encouragement were so impactful. I would also like to thank Dr. Michael Gaenzle for all the valuable expertise and insight that you have brought to this project. Your enthusiasm for microbial metabolism is contagious. Thank-you to Dr. Dan Columbus and Dr. Vera Mazurak for sharing your time and knowledge as members of my examining committee.

To past and current lab members and colleagues my sincere thanks for making the PhD journey that much richer. Dr. Janelle Fouhse, Dr. Tingting Ju, Dr. Peris Munyaka, Dr. Ben Bourrie, Dr. Andrew Forgie, Dr. Tulika Bhardwaj, Dr. Jason Grant, Dr. Arun Kommadath, Dr. Emily Herman, Dr. Kirill Krivushin, Dr. Eóin O'Hara, Tausha Prisnee, Rajibur Rahman, Camilla Marcolla, Carla Sosa, Edward Fan, Deanna Pepin, Maanasa Mudoor Sooresh, and Pravina Yadav. Thank you for all the help, the spirited discussions, and the opportunities to collaborate. But most of all, thank you for the great memories of time well spent.

I have had the pleasure of working with many brilliant collaborators in the course of this work. Dr. Paulina de la Mata, Dr. Seolin Nam, Dr. Tetske Hulsof, Dr. Theo van Kempen, and Dr. Ruurd Ziljstra – Thank you! You have each helped broaden my perspectives and knowledge base.

Last, but never least, thank-you to my wonderfully supportive family and friends. Robin, we're a long way from ANSC 484. For all you've done along the way, there are no words. Mum

& David, thank-you for all your love and support, from listening to me give unrequested biology lectures in the 90s to flying across the Atlantic in 2022 you've been there the whole way. To the many others, especially PD, KD, AG, JP, BP, RP, DB, QF – I couldn't have done it without your encouragement and support.

This work was made possible through funding support from the National Science and Engineering Council and Alberta Innovates Doctoral Scholarships, as well as through funding from the 2014 Genome Canada LSARP – Application of genomics to improve disease resilience and sustainability in pork production.

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List of Abbreviations

ABHD4	Abhydrolase Domain Containing 4, N-Acyl Phospholipase B
ADFI	Average daily feed intake
ADG	Average daily gain
ADM2	Adrenomedullin 2
AhR	Aryl-hydrocarbon receptor
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
ASNS	Asparagine Synthetase (Glutamine-Hydrolyzing)
ASV	Amplicon sequence variant
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BA	Benzoic Acid
BCFA	Branched-chain fatty acid
CHAC1	ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1
CP	Crude protein
CRC	Colorectal carcinoma
CRF	Cortocotropin-releasing factor
CYP450	Cytochrome P450
DE	Differentially expressed
dsDNA	Double stranded deoxyribonucleic acid
DSS	Dextran sulfate sodium
ETEC	Enterotoxigenic *Escherichia coli*

FDR	False discovery rate
FS	Feature Selection
GC	Gas Chromatography
GCxGC-TOFMS	Two-dimensional gas chromatography and time-of-flight mass spectrometry
GI	Gastrointestinal
GPT2	Glutamic--Pyruvic Transaminase 2
HSD	Honestly significant difference
IBD	Inflammatory bowl disease
IECs	Intestinal epithelial cells
IPA	3-indolepropionic acid
ISR	Integrated stress response
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LEfSe	Linear discriminant analysis of Effect Size
LSD	Least significant difference
MCFA	Medium-chain fatty acid
MCT1	Monocarboxylate transporter 1
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
m/z	Mass:charge ratio
NAPE	*N*-Acylphosphatidylethanolamines
NIST	National Institute of Standards and Technology
OTU	Operational taxonomic unit

PC	Phosphatidylcholine
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PERMANOVA	
PHGDH	Phosphoglycerate dehydrogenase
PSAT1	Phosphoserine Aminotransferase 1
PSPH	Phosphoserine Phosphatase
PWD	Post-weaning diarrhea
QEA	Quantitative enrichment analysis
rCCA	regularised Canonical Correlation Analysis
rRNA	Ribosomal ribonucleic acid
SAS	Statistical Analysis
S/N	Signal:Noise ratio
SCFA	Short-chain fatty acid
SWATH	Sequential Window Acquisition of all Theoretical Mass Spectra
TIM-2	TNO In Vitor Model of the Colon
TNFα	Tumor Necrosis Factor alpha

Chapter 1: Dietary Interventions for Post-Weaning Diarrhea

1.1 Introduction

Post-weaning diarrhea (PWD) is a multi-factorial disease of significant importance to swine production systems worldwide [1]. While enterotoxigenic *Escherichia coli* is the pathogen responsible for most cases of PWD, many other management and nutritional factors contribute to disease severity and prevalence [1,2]. Until recently, the use of in-feed antibiotics has been the key strategy to decrease the incidence of PWD [3,4]. With growing concerns about microbial resistance to antibiotics and changes to usage regulations, there has been a need to identify alternative strategies to manage PWD [1,3,5,6]. One area of significant focus has been in the development of dietary strategies that help address the alterations in gastrointestinal physiology and microbiota during the weaning transition.

1.2 Physiological Changes Following Weaning

Abrupt weaning at a young age is a multiple stressor event, subjecting piglets to social stress, changes in housing, and dietary change approximately three months before natural weaning [7]. At this age, piglets are physiologically and immunologically immature and the weaning transition is associated with many changes in gastrointestinal tract structure, function, and inflammatory status.

Decreased feed intake is common in newly weaned piglets, reducing nutrient availability in the gastrointestinal lumen [8]. This results in altered intestinal morphology and decreased surface area for digestive and absorptive functions [9]. Intestinal epithelial cells (IECs) are extremely metabolically active, with a high demand for nutrients obtained through first-pass metabolism [10]. This lack of nutrient availability caused by low feed intake also contributes to transient alterations in cellular turnover, gut barrier function, and local inflammatory immune cell

recruitment [11–13]. Concurrent to reduced feed intake, changes in dietary composition result in alterations in digestive enzyme secretion affecting the digestion of proteins, lipids, and carbohydrates [14–16]. All these critical changes in the gastrointestinal tract occur at an age when maternal immunity is waning and the pig's own immune system is relatively naïve [17], underscoring the need for interventions to manage this high-risk period.

The social stressors of weaning themselves may directly lead to impaired gut barrier function through corticotropin-releasing factor (CRF) and serotonin signalling in response to activation of the hypothalamic pituitary adrenal axis and elevated cortisol [18–20]. Increased CRF has been shown to alter intestinal permeability through initiation of mast cell degranulation and release of proteases and TNFα [17]. Altered serotonin signalling, is well characterized in other mammals during stress-related diarrhea and has been more recently explored for its role in diarrhea after weaning [18,19]. Serotonin release from enterochromaffin cells in the gastrointestinal tract can result in increased luminal secretions and immune cell recruitment [19]. This altered immune and secretory profile combined with a nutrient-poor luminal environment has great implications for the commensal microbes within the gastrointestinal tract and can lead to disruptions that enable pathogen expansion.

1.3 Gut Microbiota Following Weaning

With the abrupt transition from sows' milk at weaning, the gut microbiota undergoes turnover in both composition and functional capacity. During this time the dominant species adapted to milk oligosaccharides give way to those adapted to utilize plant glycans [21]. A hallmark of this period is a loss of microbial diversity, and in particular *Lactobacillus* spp., leading to a highly disrupted microbial community [7]. While some of this change is due to differences in available substrate in the gastrointestinal lumen, stress- and inflammation-induced changes

described above also contribute to the loss of microbial diversity. Reactive oxygen species and nitrous oxide generated during inflammation create an environment that is not well-tolerated by many of the anaerobic constituents of the gut, resulting in a loss of these key members of the community [22]. As these factors lessen and feed intake rebounds, new groups of microbes, such as *Prevotella* and *Clostridium* spp., come to predominate in the large intestine [23,24]. In the small intestine, *Lactobacillus* spp. rebound following this transient period of diversity loss [25,26].

However, in the period of microbiota disruption, pathobionts may expand and flourish in the gut, reaching sufficient density to cause disease [6]. Therefore, any dietary strategy that expedites the assembly of the functionally competent post-weaning microbiota can reduce disease risk and the need for antibiotic treatment [24].

1.4 Dietary Strategies

Dietary interventions for weaned pigs have generally focused on enhancing feed intake, reducing pathogen load in the gastrointestinal tract, assisting with assembly of the post-weaning microbiota, or enhancing immune and digestive functions of the gut [5,21]. Key strategies for diet formulation include reducing dietary protein, improving digestibility with enzymes, and adding ingredients such as medium chain fatty acids (MCFAs), organic acids, polyphenols, or direct-fed microbials which may control intestinal pathogens [5,6,27]. These additives aim to address different aspects of the physiological and microbial changes described above, and are used both as individual additives or in blends aimed at supporting weaned piglets.

1.5 Organic Acids

Following weaning, pigs are unable to maintain a low gastric pH sufficient for bactericidal activity and optimal protein digestion [28]. Added organic acids can be used both to prevent pathogen growth and improve protein digestibility [28,29]. Previous studies have shown the ability

of organic acids to decrease *E. coli* counts and inhibit *Salmonella typhimurium* [30–32]. However, the true antimicrobial activity of these additives *in vivo* is variable, depending on other characteristics of the luminal environment [30], highlighting a need for additional studies to link organic acids to changes in microbial metabolites and host physiology.

1.6 Medium-chain Fatty Acids

Medium-chain fatty acids are another dietary additive used to reduce gastrointestinal pathogen expansion following weaning [24]. Their antimicrobial actions are thought to be through disruption of phospholipid membranes, and they are commonly used in additive blends with other synergistic compounds [33,34]. Through this antimicrobial activity, MCFAs have been shown to provide benefits during pathogen challenge [35]. The use of MCFAs in additive blends has also been shown to improve growth and feed efficiency, as well as reduce diarrhea and improve measures of immune function [36,37]. Similar to organic acids, research to date has focused on production and disease challenge outcomes related to the use of MCFAs, and a mechanistic understanding of their effects is still needed.

1.7 Dietary Enzymes

Abrupt dietary change and low feed intake post-weaning contribute to lower secretion of digestive enzymes needed to break down and absorb nutrients during this adaptation period [14,15,38]. Concurrently, the fibre-degrading capacity of the microbiota is not yet developed [38]. Addition of dietary enzymes can help to improve the availability of nutrients to the pig and increase the availability of easily fermentable substrates to the gut microbiota, thereby improving pathogen exclusion and gut barrier function [5,39]. While studies have shown an effect of many dietary enzymes on important microbiota constituents [40–42], their mechanism of modulating the whole microbiota or microbial metabolism requires further characterization. To fully leverage these

products in the weaned pig, a better understanding of how these enzymes contribute to microbiota and host effects are needed [41,43].

1.8 Low Protein Diets

Lowering dietary protein in the period immediately post-weaning is a common practice to help reduce the risk of post-weaning diarrhea [6]. This intervention is thought to limit the amount of undigested protein utilized for fermentation and production of harmful metabolites such as ammonia [44]. However, these effects are highly dependent on overall dietary context and strongly affected by diet quality and availability of fermentable carbohydrate [45–47]. It has also been shown that these low protein diets reduce pig growth and have detrimental effects on intestinal tissue morphology [48,49]. This trade-off may not be necessary if an optimal ratio of protein and fibre in post-weaning diets could be described with considerations for supporting growth and tissue metabolism as well as diarrhea risk [45]. However, there remains much to learn about the role of these protein fermentation metabolites and the link between dietary protein and pathogen expansion to support the development of such recommendations.

1.9 Conclusion

While many dietary strategies exist as alternatives to antimicrobials for weaned pigs, there is a great deal of variation in efficacy and response when these strategies are implemented in different production settings [3]. This is likely due to the differences in factors such as gut microbiota and mucosal immunity [6]. Further understanding of the mechanisms by which these strategies work will help to delineate best practices for their application and identify factors that hamper their effectiveness. To date, less emphasis has been placed on the functional characteristics of the gastrointestinal tract that are important for management of gut health at critical life stages and how this is affected by nutrition [6].

1.10 Project Objectives

To elucidate the impacts of three of these common strategies on gut microbiota, metabolites, and host response, three studies were conducted examining the role of benzoic acid and enzymes, an additive blend with MCFAs, and ratios of protein and fibre respectively. Multi-omics techniques were utilized to provide a more wholistic picture of the impacts of these strategies on gut microbiota, microbial metabolism, and host response with a focus on functional characteristics indicative of recovery from post-weaning stress. These studies will help to inform future recommendations for feeding weaned pigs and provide important insight into the mechanistic underpinnings of responses to these common dietary strategies.

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Chapter 2: Microbial fermentation of dietary protein: An important factor in diet-microbe-host interaction

2.1. Introduction

The gut microbiome is composed of a diverse range of species, the majority of which have a mutualistic relationship with the host and, with 100 times more genes, perform metabolic functions greatly beyond those encoded in the host genome [1]. These reactions create secondary metabolites that can be beneficial or harmful. The ability of microbes to extract energy from indigestible carbohydrates and produce beneficial short chain fatty acids (SCFAs) is well established [2,3]. Microbial metabolites are also increasingly being recognized for their importance in modulating host metabolic and immune response [3,4]. Much less is known about the role that proteolytic fermentation by gut microbes plays in host health and metabolism. However, it is clear that long-term dietary patterns such as increased consumption of protein or decreased consumption of fibre can shift the composition of the microbiota, changing which taxa and fermentation pathways are most abundant [5]. These system level shifts in composition and metabolism hint at the complexity of metabolic interactions occurring in the gut microbial ecosystem, but more information is needed on the processes underlying these changes. Despite the efficiency of host digestion and absorption, some nitrogen-containing compounds in the intestine are metabolized by the microbiota. This is due both to the dispersal limitation of brush-border enzymes in the small intestine and excessive protein intake [6]. In the case of high-protein weight loss diets, protein intake may be 2-5 times greater than the daily dietary recommendations [7]. Understanding the fate of undigested dietary

protein is an important consideration in determining the effects of long-term dietary patterns on health.

Amino acids are building blocks for microbial protein, making them important for microbial growth, however, they can also be fermented as an energy source [8]. Undigested peptides are broken down by proteolytic bacteria and subsequently used either in proteolytic fermentation or to form microbial cell components. Microbial protein has a high proportion of branched-chain amino acids, though exact composition varies between bacterial strains [8]. Catabolism results in many metabolites that affect the host beyond amino acid availability. The fate of amino acids depends on ecological, and dietary factors that influence the relative amounts of proteolytic fermentation. For example, low dietary fibre may result in increased proteolytic fermentation due to the low amount of fermentable carbohydrate in the colon [9]. Shifts toward increased proteolytic fermentation can alter the relative abundance of microbial species in the gut and generate bioactive, potentially deleterious metabolic products [8].

Limited information is available about the role of proteolytic fermentation in the complex metabolic networks between gut microbes and their host. More information is needed regarding what products are generated, which species are involved or affected, and how these changes come together to affect the host. In complement to other reviews, we will describe how pathways of proteolytic fermentation, metabolites produced, and dietary pattern converge to affect health. In particular, this review will focus on compartment specific effects of proteolytic fermentation in different segments of the intestine along with metabolites such as ammonia, p-cresol, and amines that may shape health.

2.2. Proteolytic Fermentation Involves Many Metabolic Pathways:

Modeling of host and microbiota metabolic networks using genome annotation has identified 3,499 distinct reactions; of these, 1,267 are unique to the microbiota and 1,142 are shared with the host [4]. When these reactions are attributed to larger functions, the intricate interdependencies of host and microbiota metabolism become evident; Three-quarters of all pathways utilize both host and microbiota reactions [4]. This collaboration of host and microbial reactions determines the fate of dietary protein in the gut, and the overall effects on host amino acid balance and metabolism (Figure 2.1). The interrelatedness of metabolic reactions also complicates modelling of fermentation dynamics in the gut environment, limiting our current understanding of protein fermentation.

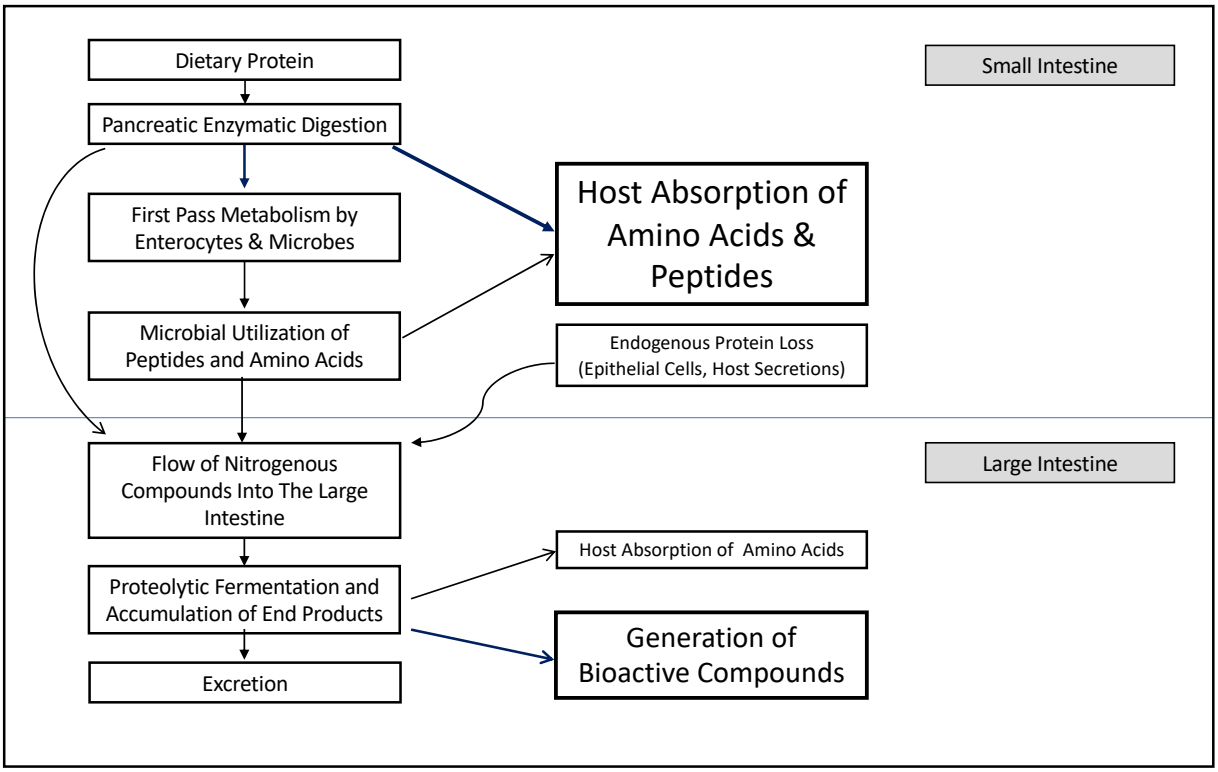

Figure 2.1. The fate of dietary protein in the gut is determined by a network of metabolic processes including both host and microbial digestion and utilization. Dominant effects for each compartment are bolded and shown with dark blue arrows.

Protein that escapes host enzymatic digestion in the small intestine can be hydrolyzed by bacteria using extracellular proteases and peptidases, resulting in free amino acids and peptides that can be taken up by the bacteria [10,11]. Culture-based experiments suggest that gut bacteria preferentially assimilate and ferment peptides over amino acids; a process which is more energetically efficient [6]. Once in the cytoplasm, amino acids can be incorporated into microbial protein or they can enter a catabolic pathway involving highly specific enzymes which perform deamination and decarboxylation followed by alpha and beta-elimination [8,11]. Deamination, the first step of the catabolic pathway, removes the amine group from the amino acid, freeing the carbon skeleton. This can be performed on single amino acids, pairs of amino acids (Stickland

reaction), or on one amino acid in combination with a non-nitrogenous compound [12]. The products generated by deamination are ammonia and keto-acids [12]. Paired amino acid catabolism, via the Stickland reaction, occurs when one amino acid is decarboxylated and the other is reduced; alanine, leucine, isoleucine, valine and histidine are preferentially reduced, while glycine, proline, ornithine, arginine and tryptophan are preferentially oxidized [13]. Ammonia generated through amino acid catabolism can be used as a nitrogen source for de novo protein synthesis or may be excreted. Keto acids proceed through decarboxylation reactions and can be used to generate short-chain fatty acids (SCFAs) including butyrate, acetate, propionate, lactate, succinate, and formate [13]. Sulfur is also liberated from amino acids during these processes [10]. Not all amino acids are equally suitable for fermentation, and differences in bacterial growth are observed when single amino acids are compared as sole energy sources in culture media. The highest growth is observed from catabolism of glutamate, arginine, glycine, serene, phenylalanine and tyrosine; though tryptophan, aspartate and alanine can also be used [6]. This complex series of reactions paired with the specific enzyme requirements for catabolizing different amino acids makes predicting the overall metabolite pool in the gut challenging. However, the available information on metabolites generated from catabolism of specific amino acids may help inform future work focused on understanding what luminal metabolites are generated from proteolytic fermentation when different levels and types of dietary protein are consumed.

2.3. Proteolytic Fermentation Produces Diverse Metabolites

Identifying metabolites generated though proteolytic fermentation in the gut lumen has been limited to date by the complexity of the luminal contents and limitations in classifying metabolites as host or microbe derived [4]. Similar to fibre fermentation, protein fermentation produces short-chain fatty acids, however these are accompanied by branch-chained fatty acids, ammonia, amines,

hydrogen sulfide, phenols, and indoles [14]. Some amino acids have characteristic metabolite profiles such as those generated for branched-chain and aromatic amino acids, which can be used as an indicator of protein fermentation in the gut [15]. Many of these products are also being identified as bioactive molecules, with roles in signalling.

While SCFAs are generated through proteolytic fermentation, total SCFA, acetate, butyrate and production are lower than what is generated from carbohydrate fermentation, while propionate production remains relatively stable [16]. These differences in SCFA abundance and profile are functionally important as butyrate provides energy to colonocytes, while acetate is thought to have important systemic effects including in reducing hypertension [3,17]. Butyrate is produced through catabolism of glutamate and lysine by bacterial species, including potentially harmful *Fusobacterium* spp. [18]. Branched-chain fatty acids (BCFAs) are reliable markers of proteolytic fermentation as they are produced exclusively through fermentation of branched-chain amino acids. The production of BCFAs increases within 24 hours on a high protein diet demonstrating how rapidly dietary protein can alter the host metabolite pool [16,19]. High-protein diets also modify the BCFA profile, increasing the cumulative production of isovalerate, which constitutes a small proportion of the BCFA pool under normal conditions [6,16]. Little is known about the effects of BCFAs on host physiology, but there is some evidence to show that they can be oxidized when butyrate is not available [10]. BCFAs are not thought to be important in colonocyte proliferation or apoptosis *in vitro*, however a lack of *in vivo* evidence limits our understanding of what effects an increase in their concentration may have. By their nature, these catabolic processes generate ammonia which can then be utilized in the host urea cycle, or must be excreted due to its toxicity [10,18]. Ammonia concentrations in the intestine result from cumulative effects of enterocyte metabolism, microbial deamination, and microbial protein synthesis [6,20]. Increased

carbohydrate fermentation and bacterial growth can decrease ammonia concentrations in the gut due to higher incorporation of nitrogen into microbial cells [21].

In addition to BCFAs and ammonia, increasing interest is being paid to other metabolic derivates of proteolytic fermentation. Some of these products are implicated in diseases including colorectal cancer, while others such as dietary polyamines play an important role in small intestine mucosal cell physiology and immune system development [4,22,23]. Polyamines are produced via a diverse set of pathways which result in the decarboxylation of amino acids [24,25]. Many amine producing species from genera including *Bifidobacterium, Clostridium, Lactobacillus, Escherichia*, and *Klebsiella*, have been identified in the gut microbiota [26]. Bacteria utilize polyamines in RNA synthesis, as structural components of cell membranes or peptidoglycan, and to protect against damage from reactive oxygen species or an acidic environment [27]. This production of amines during times of physiological stress can result in changes in bacterial pathogenicity, as well as host susceptibility to infection making these compounds candidates for further exploration with respect to their roles in gastrointestinal infection as well as carcinogenesis which is further discussed in subsequent sections [27] .

Ten putrefaction pathways generating these diverse end products have been identified thus far, and have been attributed to many of the major microbial phyla in the gut including Firmicutes, Bacteroidetes, and Proteobacteria [28]. Fermentation of aromatic amino acids may be particularly important biologically, as this generates a wide range of bioactive end products such as phenol and p-cresol (Tyrosine), or indole and skatole (Tryptophan) [4]. Probabilistic pathway construction has identified three microbial pathways catabolising tryptophan and generating a total of 10 products, six of which participate in host metabolism [4]. Evidence of the role of microbes in production of these products is demonstrated by their minute abundance in the cecal contents of germ-free mice

[4]. Microbiota metabolism of tyrosine can produce phenol, a product which is not detected in the absence of microbes [4]. These metabolites are excellent candidates for further exploration and validation to identify which microbial species may be important in generating specific metabolites *in vivo*. Currently, little is known about how changes in the microbiota impacts the production of these bioactive molecules.

2.4. Many Microbial Species Contribute to Proteolytic Fermentation

Identifying the bacterial species responsible for proteolytic fermentation has primarily used correlative methods and culture on amino acid containing media. Typically, these experiments have used branched-chain fatty acids as a marker of proteolytic fermentation. Culture of digesta obtained from the human colon suggests that isobutyrate forming species may account for up to 40% of the total anaerobes in the intestine, while abundance of isovalerate producers is more variable [6]. The relative abundance of these species may be altered in high-protein diets, where increased isovalerate production is observed [16]. Species implicated in proteolytic fermentation *in vitro* include bacteria in the genera *Clostridium*, *Fusobacterium*, *Bacteroides*, *Actinomyces*, *Propionibacterium,* as well as *Peptostreptococci* [6]. Clostridium are important for lysine and proline utilization via fermentation in the colon, while *Peptostreptococci* drive tryptophan and glutamate catabolism [29]. Aromatic amino acid metabolism reactions are thought to be primarily performed by *Enterobacter* and *Escherichia* spp. [4]. BCFA abundance has also been correlated with decreased Firmicutes and increases in unknown Bacteroidetes, as well as *Prevotella* spp., *Bacteroides ovatus*, *Bacteroides thetaiotamicron*, and *Clostridium* spp. in a TIM-2 model of high protein diets [16]. These changes occurred despite acidic fermentation conditions, which likely inhibited some bacterial proteases [6,16]. Another method, examining the presence of putrefaction

pathways in gut microbes *in silico*, have also implicated *Bacillus* spp. in protein fermentation, despite not being identified in culture experiments [28].

A newer approach, using KEGG pathway analysis of annotated human gut bacterial genomes and probabilistic pathway construction shows that Proteobacteria possess the broadest gene coverage of amino-acid reactions, though only 9% are unique to this phylum [4]. This can be explained by the many metabolic functions that are conserved across species, resulting in high functional redundancy in the microbiome [4,30]. When KEGG classification is performed on all predicted microbial reactions, the largest identified category is amino-acid metabolism (16% of all reactions), however it is important to consider that approximately 21% of reactions are unclassified, highlighting just how much work is still required to determine the true functional capacity of the gut microbiome [4]. Given the high number of unculturable species in the gut, and the simplicity of single or paired amino acid media, it is likely that not all species contributing to proteolytic fermentation *in vivo* have been identified. Likewise, the species with the greatest capacity for proteolytic fermentation cannot be identified in a non-competitive environment. Due to the differences in substrate abundance, community membership, and species richness in different locations of the gut, it is important not only to establish which species are participating but to examine how processes may differ in the small and large intestine [8].

2.5. Proteolytic Fermentation in the Small Intestine Affects Host Amino Acid Balance

Despite the fast transit time and high degree of host absorption of peptides and amino acids, evidence suggests that microbial utilization of amino acids begins in the small intestine [8,31]. A shift in ileal microbiota structure has been demonstrated in response to dietary protein levels [32]. Compared to a high protein diet (16% crude protein), moderate dietary protein (13% crude protein) decreases *Clostridiaceae* and biogenic amines in the ileum of pigs while also increasing tight

junction proteins claudin and occludin [33]. This contradicts other studies showing a beneficial effect of amines on gut function and suggests that there may be a threshold effect beyond which amine production is detrimental to gut barrier function. Polyamines are readily absorbed from the gut lumen and are important regulators of cellular metabolism, growth and proliferation [34]. However, at high concentrations amines have been shown to cause inflammation and epithelial shedding of gastrointestinal mucosa as well as disorganization of pancreatic tissue in young animals [35]. New evidence also shows that 30-50% of essential amino acids may be utilized in first-pass metabolism occurring in the small intestine; microbial utilization is thought to play a role, though the extent of utilization is not known (Figure 1) [20]. This is an important consideration as microbial and endogenous proteins resulting from first-pass metabolism are poorly absorbed once they pass the ileocecal junction [20,36]. Sequential sub-culture experiments demonstrate that, *Klebsiella* spp., *Streptococcus* spp., *E. coli*, and *Mitsuokella* spp. from the porcine small intestine utilize amino acids at an appreciable rate of 50-90% over 24 hours, which may impact overall small intestine amino acid metabolism [31]. These microbes preferentially use lysine, threonine, arginine, and glutamine for growth but also demonstrate uptake of leucine, isoleucine, valine, and histidine [31]. Importantly, this study demonstrated that the availability of specific amino acids could alter the population of intestinal bacteria that were able to grow *in vitro.* For example, *Acidaminococcus fermentans* growth could only be supported by arginine, glutamate, or histidine presence in the media [31]. A limitation of this study is the long incubation times and high concentrations of amino acids compared to what would exist in the intestine. Additionally, disappearance rather than metabolic fate of the amino acids in these mixed cultures was observed and their relative use for energy or protein synthesis was not established. Further experiments have shown that in mixed microbial cultures, overall amino acid utilization is lower

than in pure cultures of *E. coli* or *Klebsiella* spp. alone [37]. When the fate of these amino acids was examined in more detail, mixed cultures of ileal bacteria showed large amounts of lysine catabolism when compared to incorporation into microbial protein [37]. Threonine is also highly oxidized by *Klebsiella* sp. and *E. coli,* compared to mixed cultures, which is an interesting difference compared to the overall microbial community which preferentially oxidize glutamine and arginine [37]. This suggests that in pathological conditions where *E. coli* or *Klebsiella* are highly abundant in the gut, amino acids may become less available to the host, while protein fermentation by-products may contribute to disease pathology through increased inflammation. These findings also demonstrate that amino acid utilization by small intestinal microbes may be an important consideration. However, our current understanding of the physiological relevance is limited as it is unclear what percentage of required amino acids are sequestered as part of normal intestinal microbiota function. Furthermore, the culture conditions may not completely reflect the community *in vivo*. Future work feeding N^{15} labeled amino acids to animals colonized with defined communities of microbes (e.g. *E. coli* free or colonized [38]), could help to further elucidate how these findings translate in the complex gut environment. However, nitrogen recycling is a major limitation to the use of labelled amino acids. Linking proteolytic end products to specific microbes in defined communities may be a more robust way to identify markers of proteolytic pathway. Once markers of proteolytic fermentation are well established, this could also then be evaluated in humans.

2.6. Proteolytic Fermentation in the Large Intestine Generates Bioactive End-Products

In humans, carbohydrate fermentation occurs primarily in the proximal colon, resulting in a distal colon containing low fermentable substrate. Protein flowing into the large intestine may come from undigested food, bacterial cells and endogenous gut losses. These endogenous losses

include protein from enzymes, mucus, and sloughed epithelial cells [39]. The small intestine makes the largest contribution to endogenous losses, and can be affected by factors such as protein quality and fibre intake [39]. Fibre type effects the degree of endogenous losses, for example, wheat bran increases endogenous loss while no effect is seen with cellulose [40]. This effect may depend on the degree to which a given fibre type increases enzyme secretion and mucous production or decreases the digestibility of amino acids in the small intestine [39,41]. The resulting flow of protein into the large intestine may not result in detrimental accumulation of end products in these cases, as it is accompanied by microbial accessible carbohydrates. When carbohydrates are readily available, proteolytic fermentation is limited to the distal colon, where carbohydrates are depleted, creating an energy deficient environment and a pH closer to neutral [5,6]. When protein reaches the distal colon, the slow transit time and limited host absorption facilitate intense microbial proteolysis and accumulation of metabolic end products [10,36,42]. Acidic pH can greatly reduce the production of BCFAs, while in the presence of some carbohydrate more isobutyrate and isocaproate are produced [6]. In the colon, Clostridia have been identified as particularly effective at amino acid deamination via the Stickland reaction [6,11]. Through this reaction, *Clostridium* spp. are the major fermenters of lysine and proline, while *Peptostreptococcus* spp. utilize glutamate and tryptophan [29]. Preferential uptake of amino acids can be observed through the free amino acid pool, where differential abundance is observed, suggesting that some amino acids are not utilized (hydroxyproline and taurine), while others may have different fates depending on the location in the colon [6]. These alterations in free amino acid availability may have less of an impact in the colon due to the high degree of microbial utilization [18]. The effects of colonic protein fermentation on host health may therefore be considered to be based on the accumulation of bioactive metabolic products rather than changes in amino acid abundance.

2.7. Diet Affects Proteolytic Fermentation

While the metabolic pathways and microbial species involved in protein fermentation have been described in culture and modeling experiments, how these processes function under different dietary patterns still largely remains to be determined. Temporal changes in substrate availability, host absorption, and production of glycoproteins could all affect the overall degree of protein fermentation, making methodical dietary studies necessary. Certainly, it has been demonstrated that the effects of high protein diets occur quickly, with rapid changes to the microbiota and metabolites observed within 24 hours in both model systems and human trials [16,19]. Not only does high protein intake need to be considered, but the low dietary fibre intake of most western diets may complicate dietary studies due to altered abundance of fibre fermenting species in the gut [5]. Low fermentable carbohydrate intake results in low substrate availability and in turn a higher pH. This change may cause the length of the colon to more closely resemble the normal conditions of the distal colon [43]. Bacterial proteases work best at neutral pH, and are thought to be inhibited by SCFA production, therefore the relationship between dietary fibre and dietary protein may result in changes to fermentation location [23]. This is further supported by experiments comparing different types of dietary fibre, which suggest that fibre type is important in altering the relative production of branch-chained fatty acids through effects on fermentation location within the colon [45]. These results are somewhat inconsistent, where other high-protein, low-carbohydrate diets have shown an overall decrease in SCFA production, without an increased relative abundance of BCFAs [46]. This could have been due to the loss of overall species richness observed in the same study [46]. High levels of dietary protein from animal sources also selects for organisms that are more bile resistant, a function of concurrent increased fat intake [19]. Increasing bile tolerant organisms, and decreasing abundance of fibre fermenting species, suggests

that close examination of compartment-specific effects may reveal shifts in fermentation dynamics throughout the gut.

The suppression of protein fermentation by dietary fibre is thought to be due to decreasing the demand for amino acids as an energy source, and lower pH from SCFA production inhibiting proteolytic enzymes [6]. These effects result in a reduction in the amount of potentially undesirable metabolites formed [47]. Carbohydrate presence alters amino acid utilization by microbes, reducing the uptake of some amino acids such as tyrosine and increasing the use of others including valine [6]. Carbohydrate fermentation can also strongly inhibit formation of specific products such as p-cresol, which is further described below, even when degradation of their amino acid precursor (tyrosine) is still occurring [44]. This may be specific to aromatic amino acids as their complexity lends itself to a wider range of degradation pathways and metabolic end products [4]. The importance of this dietary context is demonstrated in a study comparing diet and fecal microbiota in African-Americans, a population with a high incidence of colorectal cancer (CRC), to rural South Africans, a population with low incidence of CRC. Higher levels of proteolytic fermentation products were observed in rural South Africans, however this was observed alongside increased carbohydrate fermentation and a lower incidence of colorectal polyps [48]. This finding of increased proteolytic fermentation despite lower CRC incidence suggests that the increased carbohydrate fermentation observed in rural South Africans may exert a protective effect against the effects of proteolytic metabolites on CRC development. When metabolic networks are examined, branched-chain amino acid fermentation appears to be increased on the African diet, however urinary *p*-cresol is higher in African-Americans before dietary intervention [48]. This supports the suggestion that high fibre intake may alter protein fermentation pathways and provide protective effects against inflammation and disruption of cell cycles (Figure 2.2). The mechanisms

underlying this shift in proteolytic fermentation away from deleterious metabolites remain to be elucidated. Possible mechanisms could include inhibition of particular fermentation pathways, or fermentation by specific microbial species. Likewise, fibre fermentation and resulting changes in digesta viscosity could alter interactions between metabolites and the mucosa limiting these metabolites' detrimental effects. More information on these complex metabolic interactions is needed before strong recommendations can be made.

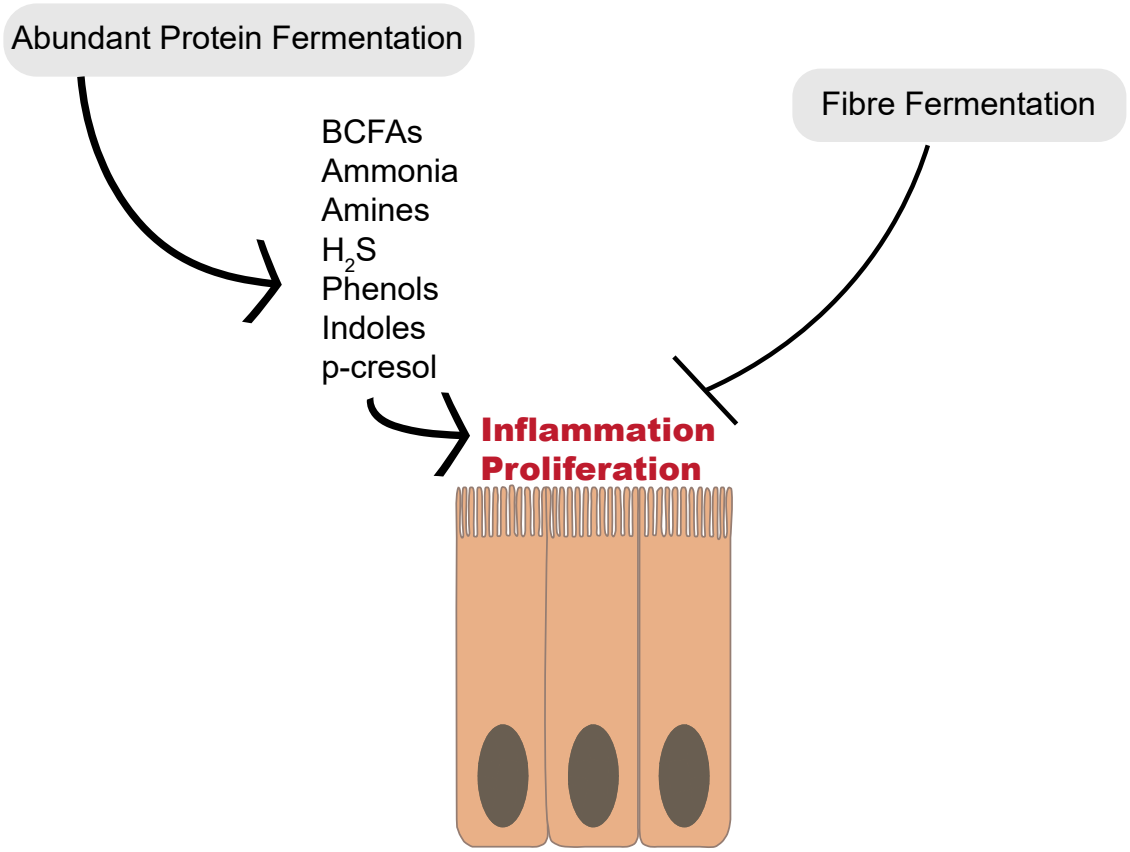

Figure 2.2. Abundant proteolytic fermentation generates a multitude of compounds that may cause inflammation and proliferation of colonocytes and in turn colorectal cancer. Increased fibre fermentation and short-chain fatty acid production appears to be protective against colorectal polyp development, even when protein fermentation products are abundant.

2.8. Protein Fermentation is an Important Consideration for Host Health

The health effects of increased protein fermentation are not entirely clear, but high protein, low carbohydrate diets for weight loss have shown to increase the proportions of phenylacetic acid from phenylalanine degradation, and *N*-nitroso compounds, raising questions about the long-term effects of these diets on colonic health [47]. In athletes, protein supplements have been shown to alter the composition of the microbiota, increasing the abundance of Bacteroidetes while decreasing *Rosburia, Blautia*, and *Bifidobacterium* [49]. However, no metabolites of microbial proteolytic fermentation were measured and while some *Bacteroides* species can ferment protein, more information is needed to ascertain if these supplements affect microbial metabolism. Another consideration is that the benefits of butyrate may also be decreased if it is produced through protein fermentation. This is due to the inflammation generated by the release of ammonia which decreases butyrate transporter expression and in turn, butyrate uptake by colonocytes [50]. Increased ammonia also decreases colonocyte oxidation of butyrate which is replaced by increased glycolysis [51]. These changes can decrease intestinal cell integrity and barrier function [10,18].

A comparative analysis of colorectal cancer patients and healthy controls found a relative enrichment of species capable of fermenting protein; putrescine, and histidine pathways were most common, and *Fusobacterium* was identified as an important differentially abundant genus [28]. This supports previous studies reporting an enrichment of *Fusobacterium* in colorectal carcinoma [52]. *Fusobacterium nucleatum* adheres to colonocytes and generates an inflammatory host response [53]. Hydrogen sulfide and ammonia are also generated by *F. nucleatum* during degradation of cysteine and production of butyrate respectively [18,54]. Sustained exposure of colonocytes to free ammonia generated during proteolytic fermentation may contribute to the development of colorectal carcinoma (CRC) [55]. Like ammonia, *p*-cresol may also cause DNA

damage and alter cell cycle, decreasing colonocyte proliferation; this may be due to its effect on colonocyte oxidative metabolism and ATP production [56,57]. This decrease in cell viability and disruption of cell cycle have also been demonstrated in atherosclerosis, alongside an increase in reactive oxygen species production [58]. Urinary *p*-Cresol is also implicated in kidney disease through its effects on endothelial cells [59]. These findings suggest that *p*-cresol is an important metabolite to consider when the detrimental effects of proteolytic fermentation are considered locally on colonocytes and systemically. However, not all proteolytic fermentation products are associated with increased CRC risk; recent studies suggest a protective effect of dietary polyamines against CRC development [60]. Further, thermolyzed protein was not shown to promote colon cancer in short-term models [61]. This underscores the importance of further examining specific metabolic pathways and products in the context of long-term dietary patterns, including consideration of dietary fibre intake.

Protein intake is also associated with increased severity of DSS induced colitis, an effect that is not seen in germ free or antibiotic treated mice (Figure 2.3) [62]. High levels of fermentable protein decreases the expression of claudins in both the distal and proximal colon, which may compensate for the detrimental effects of these metabolites [63]. Highly fermentable protein has also been demonstrated to increase expression of inflammatory cytokines in the mucosa, even if microbial composition is not different [64]. These detrimental effects of proteolytic fermentation are conflicted by other studies as reviewed by Sridharan et al., (2014), which show that indole derived from tryptophan deamination decreases intestinal epithelial inflammation and improves barrier function via tight-junctions. These local effects may be important in pathogen resistance and colorectal cancer, though much more work is needed.

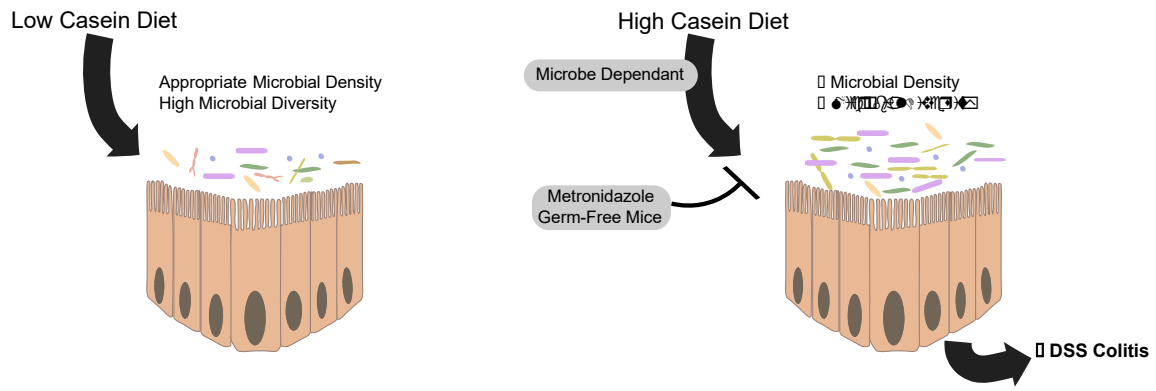

Figure 2.3. High casein diets cause an increase in microbiota density and a decrease in microbial diversity. This change in the microbiota results in an increase in DSS colitis severity. High casein diets do not have this effect in germ-free mice or if microbial density is controlled using metronidazole.

Metabolites generated through proteolytic fermentation also enter systemic circulation and travel to the liver and peripheral tissues, exerting wider effects [42]. In a twin study, metatranscriptome analysis of the microbiota shows decreased expression of genes associated with amino acid degradation pathways in microbiota from obese individuals compared to their corresponding lean twins [65]. This decreased capacity for degradation could lead to an increase of these amino acids in systemic circulation, particularly if high levels of protein are consumed and could be detrimental for health. These changes in amino acid parallel the increase in circulating branched-chain and aromatic amino acids seen in type-2 diabetes mellitus (T2DM) and insulin resistance, suggesting that altered fermentation may be important in T2DM [66]. Examination of plasma metabolites in a well characterized population of obese adolescents showed a strong correlation between higher plasma levels of BCAAs, tryptophan, lysine, and glutamate and non-alcoholic fatty liver disease [67]. In the same study, baseline plasma valine was identified as predictive of liver fat accumulation over the following two years [67]. Though not completely understood, this is suggestive of changes in intestinal permeability or microbial amino acid

synthesis which may occur as a result of altered microbial populations seen in T2DM and metabolic disease. Another factor that should not be overlooked is *de novo* synthesis of amino acids. When adults consume a protein-adequate diet, microbially produced amino acids have been shown to be a significant contributor to the plasma amino acid pool [68]. While some absorption of amino acids may be possible in the large intestine, there is a limited body of evidence to date to suggest that this absorption is physiologically relevant [36]. More work is needed to determine the relative amounts of microbially produced amino acids reaching systemic circulation from the large or small intestine and how this may be altered in high protein, low fermentable carbohydrate diets.

The role of proteolytic fermentation in health goes beyond metabolic disease, as metabolites of aromatic amino acid metabolism are capable of binding the aryl hydrocarbon receptor (AhR), an important transcription factor which may implicate them in endocrine regulation, cytokine signalling and, unlike free ammonia or p-Cresol, they may decrease cancer development [4,69]. Indole in particular, may have important effects on the nervous system and may enhance neurodevelopmental or psychiatric diseases [70,71]. In cases of a moderate and chronic overproduction of indole by the gut microbiota, rats display increased anxiety-like behaviour [70]. Indole has also been shown to be protective in mouse models of multiple sclerosis, further demonstrating its importance in connecting the gut microbiota to health outcomes in the central nervous system [72]. Indole is only produced when tryptophan is in its free amino acid form, highlighting the need for consideration of how overall diet and microbiome affect the production of important bioactive molecules from dietary protein.

2.9. Discussion

Proteolytic fermentation is a highly networked process that can exert many effects on the host. The changes in proteolytic fermentation based on fibre availability suggest that examining the role

of protein fermentation on health must also consider the carbohydrate requirement of the gut microbiota [5]. While different effects of carbohydrate type on proteolytic fermentation have been observed [45], more metabolite level information from the proximal colon and small intestine is necessary to truly understand the significance of these differences. Measurements of fecal components include a large contribution of bacterial secretions and cellular components, and therefore may not represent the changes occurring further up in the gut [6]. Untargeted mass spectrometry methods can identify compounds with increasing specificity and efficiency, however understanding the health implications of the products detected is limited. There is a need for more metabolome data from different gut compartments in healthy vs. disease states, similar to the work done in Crohn' Disease [73]. Likewise, predictive studies are limited by the accuracy of KEGG databases where ambiguous or missed pathway assignment can interfere with the accuracy of prediction [4]. This highlights the need for further studies to elucidate community-wide changes in microbiota and metabolites associated with proteolysis and to further determine which species are implicated as well as how these networks come together at the level of the whole community.

The work described previously suggests many avenues to further explore the role of proteolytic fermentation in health. For example, the differences in amino acid utilization between some opportunistic pathogens (*E. coli* and *Klebsiella* sp.) in the small intestine provides an interesting avenue for exploration of how dietary amino acid profile may be an important consideration in disease states. To date, there is no consensus model of gut microbiota metabolism, and experiments trying to identify microbial proteolytic products have used germ free mice as a comparison [4]. This approach does not allow for evaluation of which pathways are favored in a colonized gut. There is a need to pair metabolic pathway predictions on individual species and communities with validation experiments that discriminate products of microbial metabolism or

shared host-microbial metabolism from host products, as not all predicted pathways may occur in the reality of the gut environment. For example, host-only predicted pathways for aromatic amino acid degradation were not detected upon validation in a germ-free model, suggesting that, these pathways are not favored in the complex host-microbe metabolic network [4]. A search of the literature also revealed no studies examining the potential toxicity of protein metabolites to other microbes *in vivo*. Given the ability of other microbial products to limit the growth of community members, it seems plausible that some species may be negatively affected by the presence of proteolytic fermentation products. This could mean that alterations in microbial species abundance is due not only to changes in available niches, but also due to species loss from toxic products.

Considering the intricate interactions within the microbial community and with the host, more information is needed that explores beyond microbiota composition and cross-sectional studies to examine specific changes in microbial species and metabolites when dietary proteolytic fermentation is occurring. This missing information will strengthen our understanding of the mechanisms by which dietary change alters the functional profile of the microbiome and in turn affects host health and metabolism.

2.10 References

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Chapter 3: Dietary benzoic acid and supplemental enzymes alter fibre-fermenting taxa and metabolites in the cecum of weaned pigs

3.1 Introduction

To decrease the use of antibiotics in pork production, growth-promoting antibiotics are replaced with other nutritional and management strategies that maintain animal health and promote growth [1]. Two such strategies are the inclusion of organic acids, which may control growth of pathogenic bacteria in the gut, and enzymes that increase nutrient digestibility of feed [2].

Benzoic acid is known to exert antimicrobial properties during feed storage and may also compensate for the limited ability of pigs to achieve a bactericidal stomach pH immediately after weaning, preventing pathobiont expansion and post-weaning diarrhea [3–5]. In particular, a decrease in *Escherichia coli* counts in cecal digesta and feces have been observed in response to dietary inclusion of benzoic acid [6,7]. This is consistent with *in vitro* studies showing inhibited growth of pathogens, *E. coli* O157:H7 and *Salmonella typhimurium*, and commensal strains of *E. coli* and *Lactobacillus* [8,9]. However, these *in vitro* results may overestimate the antimicrobial activity of benzoic acid in the presence of digesta [8]. Additionally, there is limited understanding of the microbial mechanisms by which this additive may affect host physiology through changes in microbial metabolism in the cecum.

Supplementation with feed enzymes increases diet digestibility through reducing anti-nutritional factors and breaking down plant cell wall components; this may be particularly useful for weaned pigs as secretion of digestive enzymes and fibre-degrading capacity of the gut microbiota are not yet fully developed [10,11]. Increased diet digestibility may lead to reductions in diarrhea incidence and duration through improved gut barrier function and pathogen exclusion [11,12]. To date, information on effects of these changes in nutrient availability on the microbiota

of weaned pigs is limited. Previous findings show fecal lactobacilli counts increased and coliforms decreased in response to enzyme supplementation [13,14]. More recent studies utilizing 16S rRNA sequencing have shown an effect of feed enzymes such as β-glucanase and xylanase on the small intestine microbiota, increasing the presence of important microbiota constituents such as *Lactobacillus* and decreasing the abundance of pathobionts such as *Campylobacter* [15–17]. The use of 16S rRNA sequencing has also shown an alteration in the cecal microbiota of growing pigs [18]. However, the combination of cecal microbiota sequencing and metabolomics allow for further discernment of how previously described changes in digesta viscosity and nutrient availability may contribute to these effects [17,19].

To gain insight how these feed additives impact the gut microbiome and act as a possible mechanism for improved performance in young pigs, the effects of supplementation of benzoic acid or feed enzymes on microbial community composition (16S rRNA sequencing) and metabolism (untargeted two-dimensional gas chromatography and time-of-flight mass spectrometry (GCxGC-TOFMS metabolomics) were evaluated. The intent of this study was to gain a further understanding of the impact of benzoic acid and dietary enzymes on microbial community and metabolism using an untargeted approach. The use of metabolomics can effectively identify diet-induced alterations in microbiota function and therefore further our understanding of the effects of feed additives within the gastrointestinal tract [20].

3.2 Materials and Methods

All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009). The animal protocol was approved by the University of Alberta

Animal Care and Use Committee for Livestock and conducted at the Swine Research and Technology Centre of the University of Alberta (Edmonton, AB, Canada).

Diets

To evaluate the effects of benzoic acid (BA), feed enzymes (ENZ) or both (BAE), mash diets were formulated using a base of 28.5% wheat, 27.0% barley, 15% soybean meal, 15% canola meal, 2.5% soy protein concentrate, 5.0% lactose, 2.0% fish meal, and 0.5% canola oil plus minerals, vitamins, synthetic amino acids, and 1,000 U/kg phytase (DSM Nutritional Products Canada Inc., Ayr, ON, Canada). Growth performance and diarrhea data from this pig population are reported elsewhere [21]. This basal diet was served as control diet (CON). No antibiotics were included in any diet. Benzoic acid was supplemented in diets BA and BAE at 0.5% (VevoVitall®, DSM Nutritional Products Canada Inc., Ayr, ON, Canada). Three feed enzyme additives (Ronozyme® VP (CT), Ronozyme® MultiGrain (GT), and Ronozyme®A (CT); DSM Nutritional Products Canada Inc.) were used in diets ENZ and BAE constituting a total of 0.045% of each diet. These enzymes provided 250 U/kg xylanase, 100 U/kg β-glucanase, 100 U/kg cellulase, and 80 U amylase. Enzyme data were based on laboratory analyses following diet preparation.

Experimental Design and Housing

Piglets were weaned at 19 ± 1 days of age. Following a seven-day adaptation period, 160 (Large White/Landrace) × Duroc piglets were randomly assigned to one of 4 test diets (n = 40/treatment). Groups were balanced for sex and body weight. Feed and water were provided ad libitum throughout the experimental period using a four-space self-feeder (model N4-424; Crystal Spring, MB, Canada) and an adjustable height nipple drinker. Pigs were housed in groups of four in floor level pens (1.1 m x 1.5 m) with plastic deck flooring and solid polyvinyl chloride partitions. Animals were grouped by diet in with a total of 10 pens per treatment group. Pens were distributed

in 3 nursery rooms in a randomized complete block design. Room temperature was controlled by an automated room environmental controller and set to 32ºC at weaning with a gradually step-down of 1-2ºC per week. Artificial light was provided using eight light fixtures equipped with two 40-watt fluorescent light bulbs in each nursery room. Photoperiod was maintained using a timer and maintained at 12 hours of light (0700 – 1900 h) and 12 hours of darkness.

Sample Collection

Diarrhea was recorded daily for each using a fecal consistency scoring system from 1-8 as follows: **1**: very hard and dry, often pelleted, requires much effort to expel from body, no residue left of ground when picked up; **2**: firm, but not hard, pliable, segmented appearance, little or no residue left on ground when picked up; **3**: log-like, little or no segmentation visible, moist surface, leaves residue but holds form when picked up; **4**: very moist/ soggy, distinct log shape, leaves residue and loses form when picked up; **5**: very moist but has distinct shape, present in piles rather than logs, leaves residue and loses form when picked up; **6**: has texture, but no defined shape, occurs as piles or spots, leaves residue when picked up; **7**: watery, no texture, flat, occurs as puddles; **8:** watery with little to no color. Diarrhea incidence was calculated weekly as the percentage of days in which feces with a score of 6 or greater was observed in the pen. To measure growth performance, feed intake (measured as feed disappearance) was collected for each pen and pigs were weighed weekly. A description of the statistical analyses used for performance and diarrhea measurements has been previously reported (le Thanh et al., 2018). At the end of the experimental period, 40 piglets (n = 5/sex/treatment) of average body weight for their pen were selected for blood and digesta collection. Prior to euthanasia, pigs were weighed and sedated by injection of azaperone (Stresnil, 6 mg/kg BW, Vétoquinol N.-A. Inc., Lavaltrie, QC, Canada) and xylazine (Rompun, 2.2 mg/kg BW, Bayer, Mississauga, ON, Canada), followed by 5% of

isoflurane gas anesthetic. Blood was collected by jugular venipuncture following sedation. Pigs were euthanized by intracardial injection of 106 mg/kg BW of sodium pentobarbital (Euthanyl, Biomeda, Cambridge, Ontario, Canada) and exsanguination. Following euthanasia, pigs were placed in dorsal recumbency, their midline sterilized, and an incision made. The cecum was identified and immediately clamped to prevent mixing of digesta prior to removal. Once excised, cecal digesta was collected into 3 x 2 mL microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -80°C until further analysis. The remaining freshly collected cecal digesta was homogenized, and digesta pH was measured by inserting the electrode of a calibrated portable pH meter (Accumet Basic AB15, Fisher Scientific Company, Ottawa, ON, Canada) into the collected samples.

Microbiota Composition Analysis

Total DNA was isolated from cecal content using the QIAmp DNA Stool Mini kit (Qiagen, Montreal, QC, Canada) with the addition of a bead beating step [22]. Bead beating was performed using FastPrep-24™ (MP Biomedicals, OH, USA) homogenizer at 6 m/s for 45 seconds. Following extraction, DNA concentration was measured using a Quant-iT PicoGreen dsDNA kit (Invitrogen, CA, USA). Amplicon libraries of the V3/V4 region of 16S rRNA were constructed according to Illumina protocol (16S Metagenomic Sequencing Library Preparation) using the following primers:

Forward:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'

Reverse:

5'-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

Sequencing was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) and all raw sequences were uploaded to the National Center for Biotechnology Information Sequence Read Archive (BioProject accession number: PRJNA875948). Raw sequence data was analyzed using the QIIME2 pipeline (2022.2) [23]. Demultiplexing, quality filtering, denoising and chimera removal were performed using the DADA2 plugin [24]. Taxonomy was assigned using the naïve bayes classifier trained on the SILVA database (138) with a cut-off at 99% identity [25–27]. Diversity metrics for alpha diversity were calculated on data rarefied to a sampling depth of 1900 reads/sample using the core-metrics method and visualized within the QIIME2 pipeline. Alpha diversity was examined using both Faith's Phylogenetic Diversity and Shannon indices and compared using a Kruskal-Wallis test. Beta diversity metrics were generated using the diversity feature on unrarefied data where singletons and low-read samples (read depth < 2300) were removed and visualized by PCoA in the phyloseq package for R [28]. Differences in beta diversity were compared by PERMANOVA within the QIIME2 pipeline. Differentially abundant taxa were identified using the Linear discriminant analysis of Effect Size (LEfSe) Galaxy module [29]. Significant differences were defined as $P < 0.05$, while trends were defined as $P < 0.10$.

Metabolomics of Cecal Digesta

Sufficient volumes of digesta were obtained from 35 piglets to conduct GCxGC-TOFMS on cecal content (n = 8-9/group). 1-2 samples per group were compromised in the sampling and storage process, therefore were not included. An aliquot of 250 mg of wet sample was lyophilized for 72 hours, and the freeze-dried samples were stored at -80°C until the analysis.

The freeze dried cecal samples were weighed out (41±1.5 mg) and extracted using an 80% methanol (>99.9%, Millipore-Sigma, Oakville, ON, Canada) solution that was prepared with deionized 18.2 MΩ water (Elga PURELAB flex 3 system, VWR International, Edmonton, AB,

Canada). The dried sample was extracted using 2-mL Eppendorf tubes (Eppendorf Canada Ltd., Mississauga, ON, Canada). The sample was vortexed (Benchmixer V2, Benchmark Scientific, Sayreville, NJ) for 3 min, centrifuged for 15 min at 10,000 rpm, and 50 µL of the supernatant was transferred to a 2 mL gas chromatography vial (Chromatographic Specialties Inc, Brockville, ON, Canada) and dried under nitrogen (Praxair Canada Inc., Edmonton, AB, Canada) using a 099A EV2412S Glas-Col Heated Analytical Evaporator (Cole-Parmer, Quebec, QC, Canada) at 50 °C. A volume of 100 µL of toluene (Millipore-Sigma, Oakville, ON, Canada), dried previously with anhydrous sodium sulfate (Millipore-Sigma, Oakville, ON, Canada), was added to the sample vial and dried under nitrogen to remove the residual moisture. Methoxyamine toluene (50 µL of 20 mg/mL, Millipore-Sigma, Oakville, ON, Canada) in pyridine toluene (Millipore-Sigma, Oakville, ON, Canada) solution was added to the dried sample, samples were incubated at 60ºC for 60 min. Following this, 100 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide + 1 % chlorotrimethylsilane (Fisher Scientific, Edmonton, AB, Canada) was added to each sample, incubated 60ºC for 30 min. The derivatized samples were transferred into GC vial with an insert (Chromatographic Specialties Inc, Brockville, ON, Canada) and were subjected to the GC×GC-TOFMS analysis.

A Leco Pegasus 4D GC×GC-TOFMS (Leco Instruments, St. Joseph, MI) with a 30 m × 0.25 mm × 0.25 µm Rtx-5MS (Chromatographic Specialties Inc, Brockville, ON, Canada) columns as first dimension and a 1.7 m × 0.25 mm × 0.20 µm SLB-IL59 (Supelco, Millipore-Sigma, Oakville, ON, Canada) column as the second dimension were used. Helium (5.0 grade; Praxair, Edmonton, AB, Canada) was used as the carrier gas at a constant flow of 2 mL/min. One microliter of the sample was injected in both the splitless (inlet purge time of 80 s) and split (1:40) mode with an inlet temperature set at 270°C. The primary GC oven was programmed from 70°C, held for 5 min, and then ramped at 9.7°C/min to 280 °C with a final hold of 15 min, with a total analysis time

of 41.65 min. The secondary oven and the modulator were programmed to have a constant offset of +10ºC relative to the primary oven, and the secondary oven temperature respectively. The modulation period was 2.0 s with 0.4 s hot pulse and 0.6 s cold time. The mass spectrometry (MS) transfer line temperature was set at 270°C. Time-of-flight mass spectrometry parameters were: electron energy of -70 eV; acquisition rate of 200 Hz; the mass range of m/z 25-900; detector voltage with the optimized voltage offset of 200 V; ion source temperature of 200ºC; the solvent delay time of 300 s.

All GC×GC-TOFMS data were processed using ChromaTOF® (v.4.72; Leco) equipped with the US National Institutes of Standards and Technology MS database (NIST MS Search 2.0). The baseline offset was set to 0.9 above the middle of the noise. The data were processed with a peak-finding threshold of S/N 100:1, the minimum S/N ratio for sub-peaks to be retained was set at 6, the mass spectral match required for the sub peaks to be combined was set at 650. The peak widths for peak picking criteria were set to 12 s in the first dimension and 0.15 s in the second dimension.

Chromatograms were normalized by sample dry weight and aligned based on mass spectra and retention time using the statistical compare function in the ChromaTOF® software. The aligned peak table was then exported as a .csv file for further data analysis in MATLAB® R2017b (The Mathworks Inc., Natick, MA). The dataset was autoscaled and multivariate statistical analysis was performed using PLS Toolbox (R8.5.2; Eigenvector Research Inc., Wenatchee, WA) in MATLAB. An in-house algorithm was used to perform feature selection (FS) and model optimization on the peak table. This algorithm is a two-step hybrid backward elimination/forward selection approach that relies on cluster resolution as a model quality metric/objective function [30–32]. Principal component analysis (PCA) was used to visualize differences between

treatments. PCA models were performed using PLS Toolbox 8.5.2. Compound identification of selected features was manually performed in the Pegasus ChromaTOF software through forward and reverse similarity indices and comparison of retention indices (Tables 3.1, Supplementary Tables A1 and A2).

The normalized data from the two-step FS was imported into Metaboanalyst 4.0 for further analysis. Data were range-scaled, and a one-way ANOVA performed to identify individual compounds that differed among the four diets with a false discovery rate (FDR)-adjusted $p > 0.05$. Metabolites identified through feature selection between each diet and the control were also visualized using the heat map function.

3.3 Results

Performance and Health

Comprehensive performance data on the full group of pigs is published elsewhere [21]. In summary, pigs fed the BA diet had a greater daily feed intake and gained more weight over the experimental period than pigs fed the control diet (average daily gain 482 versus 435 g/d*; P* = 0.037); there were no differences in growth or feed intake for the other diets [21]. A time effect on diarrhea incidence was observed for all groups, with high incidence in weeks one and two of the experiment followed by a decline in week three (40, 56, and 22 % respectively; $P < 0.001$). Pen-level diarrhea incidence in week two (day 8-14) decreased 20% in pigs fed BAE compared to the control diet ($P = 0.047$) [21], indicating earlier resolution of post-weaning diarrhea in pigs fed BAE than CON the first weeks following weaning.

Cecal Microbiota

Dietary inclusion of BA alone resulted in decreased alpha-diversity of cecal microbiota (Figure 3.1). Using Faith's Phylogenetic Diversity and Shannon indices and a Kruskal-Wallis test, cecal α-diversity was different for the BA versus CON diet (Faith's PD $P = 0.041$, Shannon $P = 0.041$) and showed a statistical trend compared to the ENZ diet (Faith's PD $P = 0.086$, Figure 3.1b). Analysis of β-diversity to determine the effect of additives on microbial community composition and structure, revealed that only the addition of benzoic acid had a significant effect on measures of overall community composition compared to CON animals (Unweighted Unifrac $P = 0.047$, $R^2 = 0.07$; Figure 3.2). Further, the cecal microbiota of BA pigs tended to be different than those fed a diet which also contained enzymes (BA versus BAE, Unweighted Unifrac $P = 0.084$). The inclusion of enzymes (ENZ or BAE) did not affect community-level measures of cecal microbiota composition compared to the control diet. Only three differentially abundant features (Figure 3.3a) were observed after multiple-inference correction in the cecum of pigs fed benzoic acid. These features, which were decreased in both BA and BAE animals, belong to the genus *Streptococcus* and family *Erysipelotrichaceae*. Ten discriminative features were identified with higher relative abundance in pigs fed diets containing enzymes (Figure 3.3b). Features with increased abundance were classified to the bacterial family *Prevotellaceae* and order Bacteroidales.

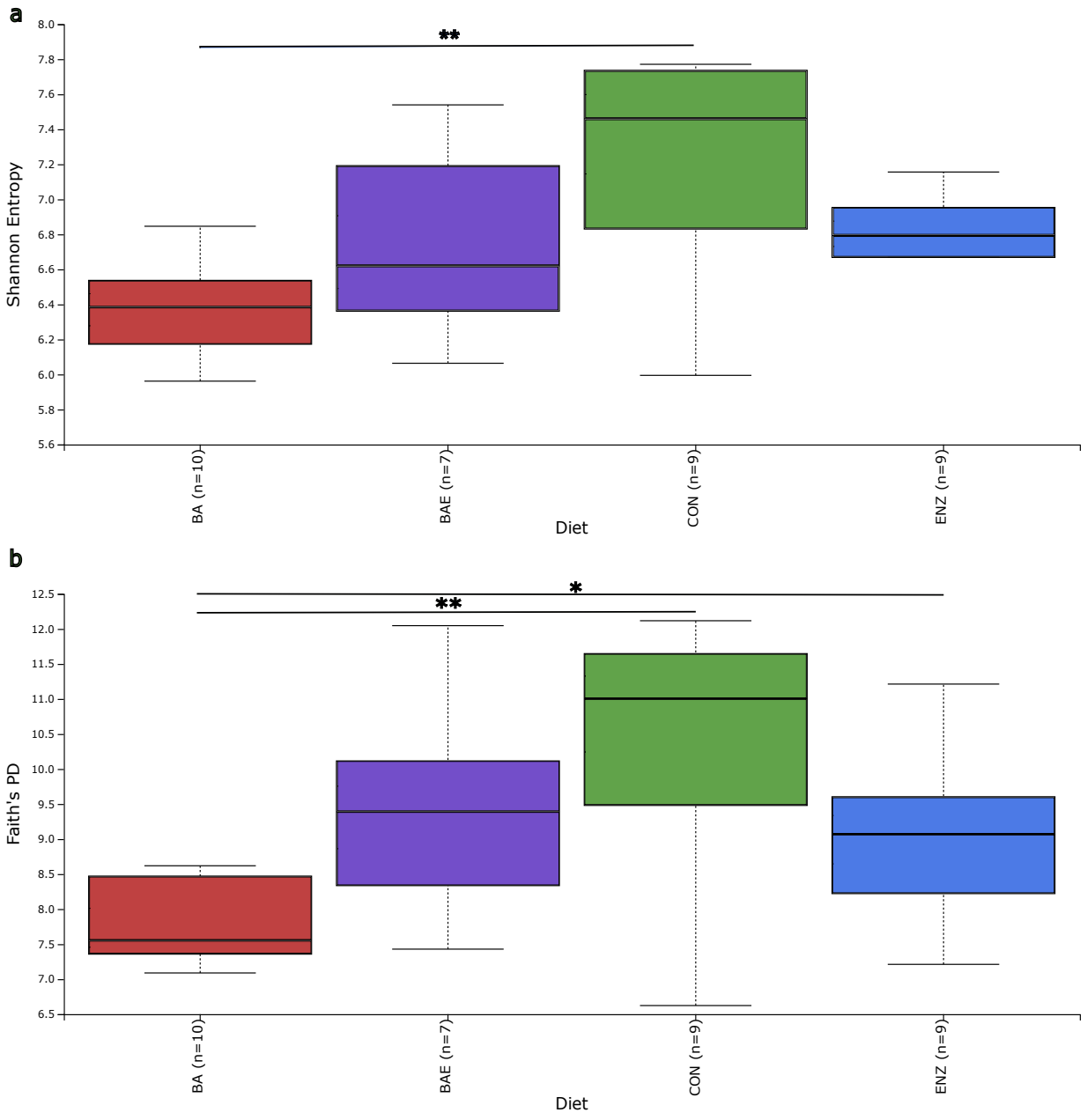

Figure 3.1. Boxplots of alpha diversity measurements for the cecal microbiota of pigs fed a control diet (CON) or diets containing benzoic acid (BA), enzymes (ENZ), or both (BAE). Alpha diversity was measured using Shannon and Faith's Phylogenetic Diversity indices and tested using a Kruskal-Wallis test. Significant differences ($P < 0.05$) and trends ($P < 0.10$) are denoted with ** and * respectively.

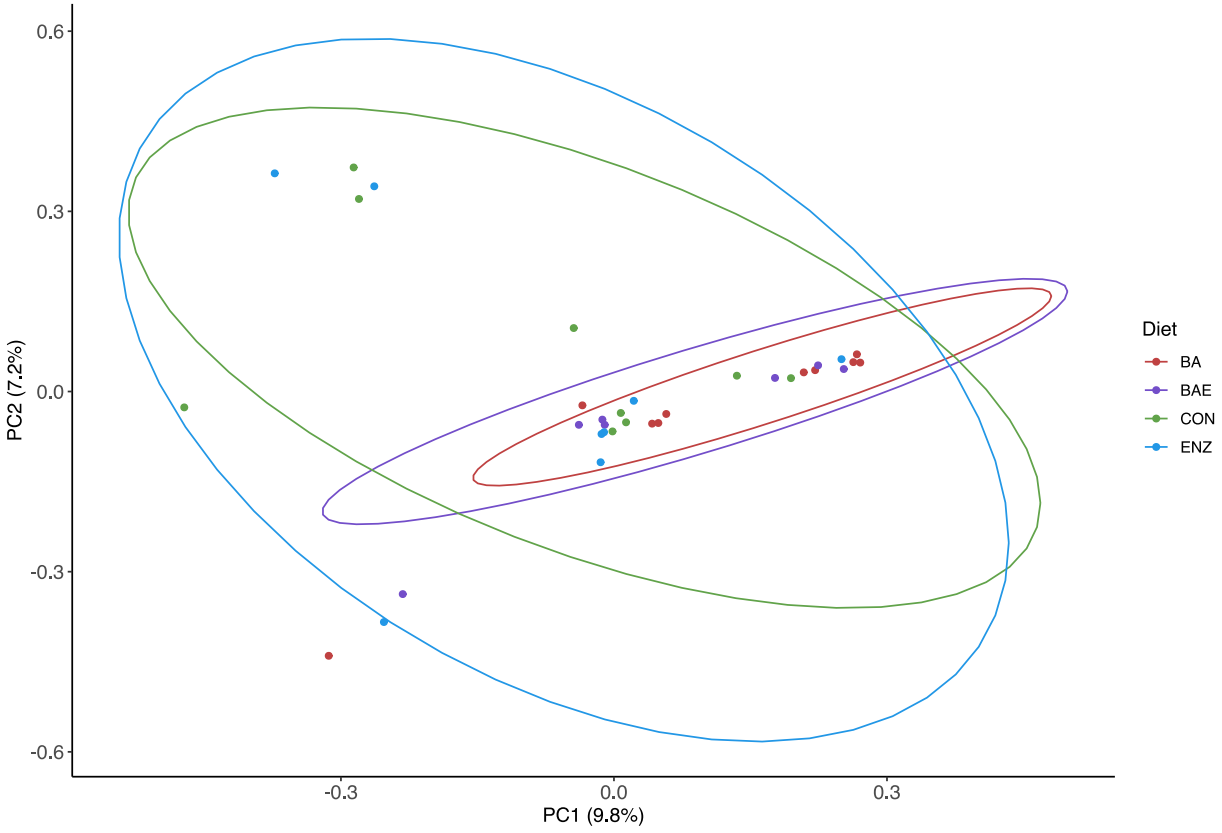

Figure 3.2. PCoA of the cecal microbiota of pigs fed one of four experimental diets. PCoA was generated using Unweighted Unifrac dissimilarity and differences in β-diversity were tested by PERMANOVA. Benzoic acid (BA) exerted a significant effect on microbiota composition compared to a control diet (CON) ($R^2 = 0.07$; $P = 0.047$) but did not affect dispersion, while no significant effect was shown for either dietary enzymes (ENZ) or both benzoic acid and enzymes (BAE). Dispersion was not significantly different between groups ($P = 0.86$).

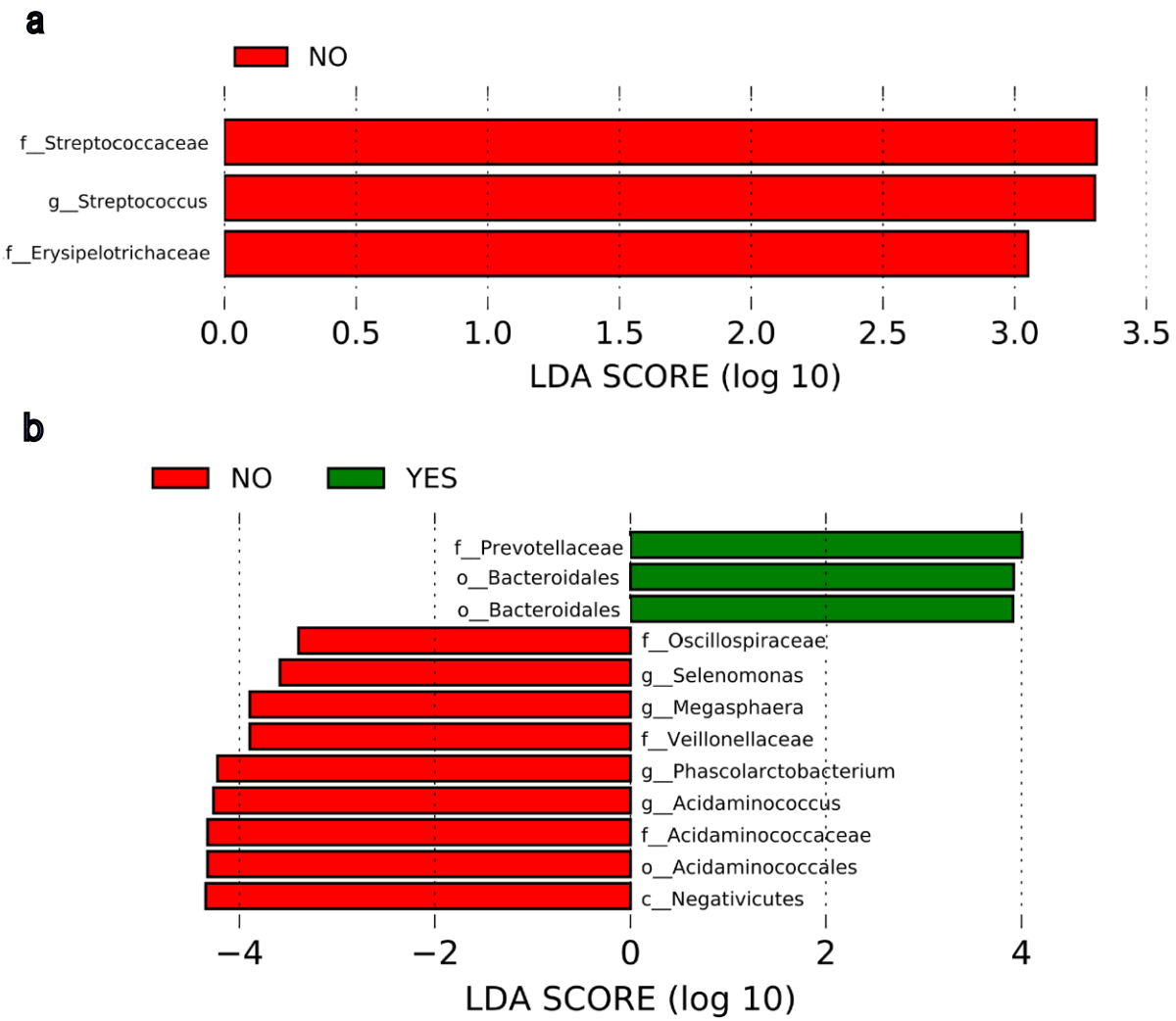

Figure 3.3. Differently abundant taxa observed in the cecal microbiota of pigs fed diets containing a. benzoic acid (YES) or not (NO) and b. dietary enzymes (YES) or not (NO). Differentiating taxa were identified using the LEfSe galaxy module and tested for significance using a Kruskal-Wallace test. Lowest classification level for each taxon was determined through taxonomy assigned using the SILVA database. Lowest classification level is denoted next to the OTU name as g_ for genus, f_ for family, o_ for order, and c_ for class.

Metabolomics of Cecal Digesta

A typical contour plot of a cecal sample analyzed using GC×GC-TOFMS is shown in Figure 3.4. Each sample presented about 3790 peaks detected with a signal/noise ratio (S/N) > 100. All chromatographic peaks were searched against the mass spectra library database. The compound name was tentatively assigned for peaks with a library match score greater than 650 (where 1000 is a perfect match) and the retention index match (absolute difference between the library and the experimental retention index values) less than 45 units. For the compounds that did not meet these criteria, the compound name was not assigned but instead, the analyte number was given.

Chemometric investigation was performed using an in-house FS protocol applied to the aligned and normalized peak table. Using the two rounds of FS, 17 compounds were selected as the metabolites responsible for making separation of all four dietary treatments (Table 3.1). Principal component analysis (PCA) was employed to model the resulting data matrix with 17 compounds and to visualize the clusters on the PCA score space. Figure 3.5a shows the PCA score plot where the four diets are grouped. Two principal components explained 39.84% of the variability of the data. Principal component 1 drives separation between control and benzoic acid diets versus feed with enzymes and benzoic acid plus enzymes diets. Meanwhile, principal component 2 separates control and feed with enzymes versus benzoic acid and benzoic acid plus enzymes diets. In Figure 3.5b, the PCA biplot shows the relationships between the diets and the peaks driving separation. Benzoic acid is related to the samples that contained benzoic acid; a sugar is related to the diet with enzymes.

Table 3.1. Compounds identified via feature selection as driving separation between all four experimental diets. For identified compounds, library match scores, retention index match (Ri_{lib}-Ri_{exp}) and CAS identification number are provided.

Metabolite Name	Count	Average Area	Quant Mass	Library Match	Retention Time 1, s	Retention Time 2, s	$\lvert Ri_{Lib}-Ri_{exp}\rvert$	**CAS**
Analyte 4828	42	60310	222		1658	0.900		
Analyte 4433	20	14065	203		1505	1.061		
Analyte 1837	8	55540	268		888	1.443		
Analyte 4681	39	390116	180		1589	1.317		
3-Hydroxyisobutyric acid	30	64137	177	881	529	1.358	7.7	55530-42-2
Sugar C36	13	64106	452		1562	1.035		
Benzoic Acid	50	4706820	179	952	614	0.059	9.9	2078-12-8
Analyte 1798	49	56981	265	650	872	1.344	10.4	51642-61-6
Analyte 4345	39	27438	297		1480	1.093		
Analyte 4199	50	254341	281		1432	1.628		
Analyte 1024	23	7534	164		676	1.690		
Analyte 2291	46	42199	255		986	1.555		
2-Ketovaleric acid	30	23493699	147	888	698	1.222	16.4	n/a
1-Monopalmitin	10	13035	385	698	1516	1.030	24.1	1188-74-5
Analyte 4690	40	51400	268		1591	1.034		
1,2-Propanediol	26	152089	117	882	332	1.181	10.6	17887-27-3
Analyte 2216	31	17369.3	157		969	1.228		

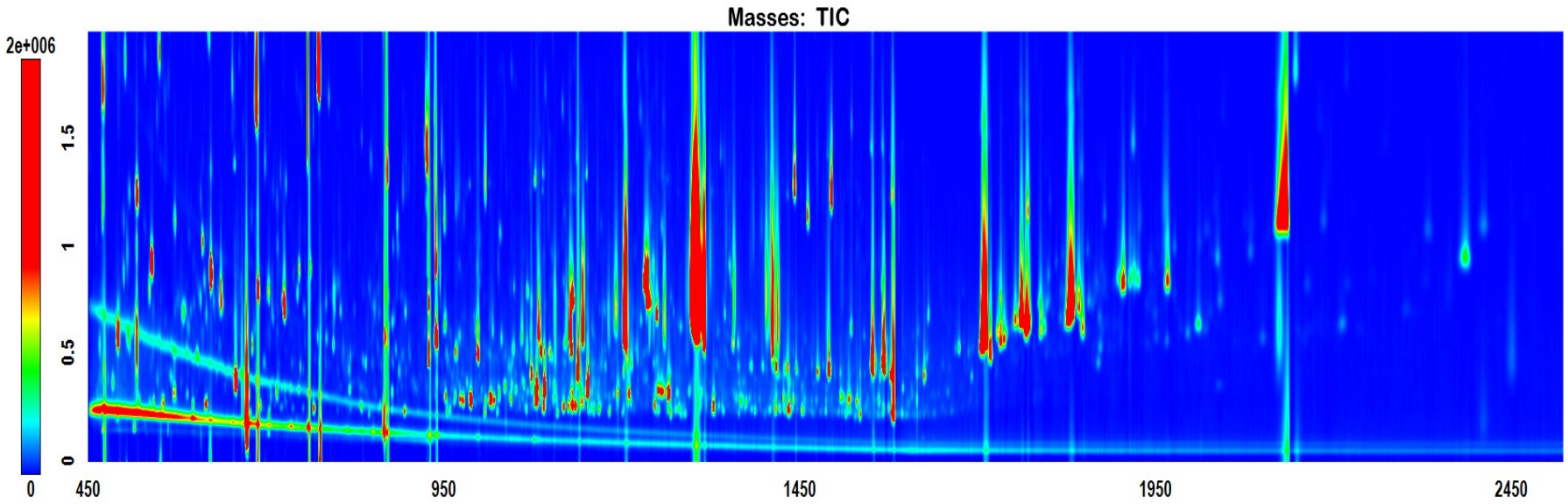

Figure 3.4. The total ion current chromatogram of a typical cecal sample. Axes are in seconds, and a colour scale gives the intensity of the peaks.

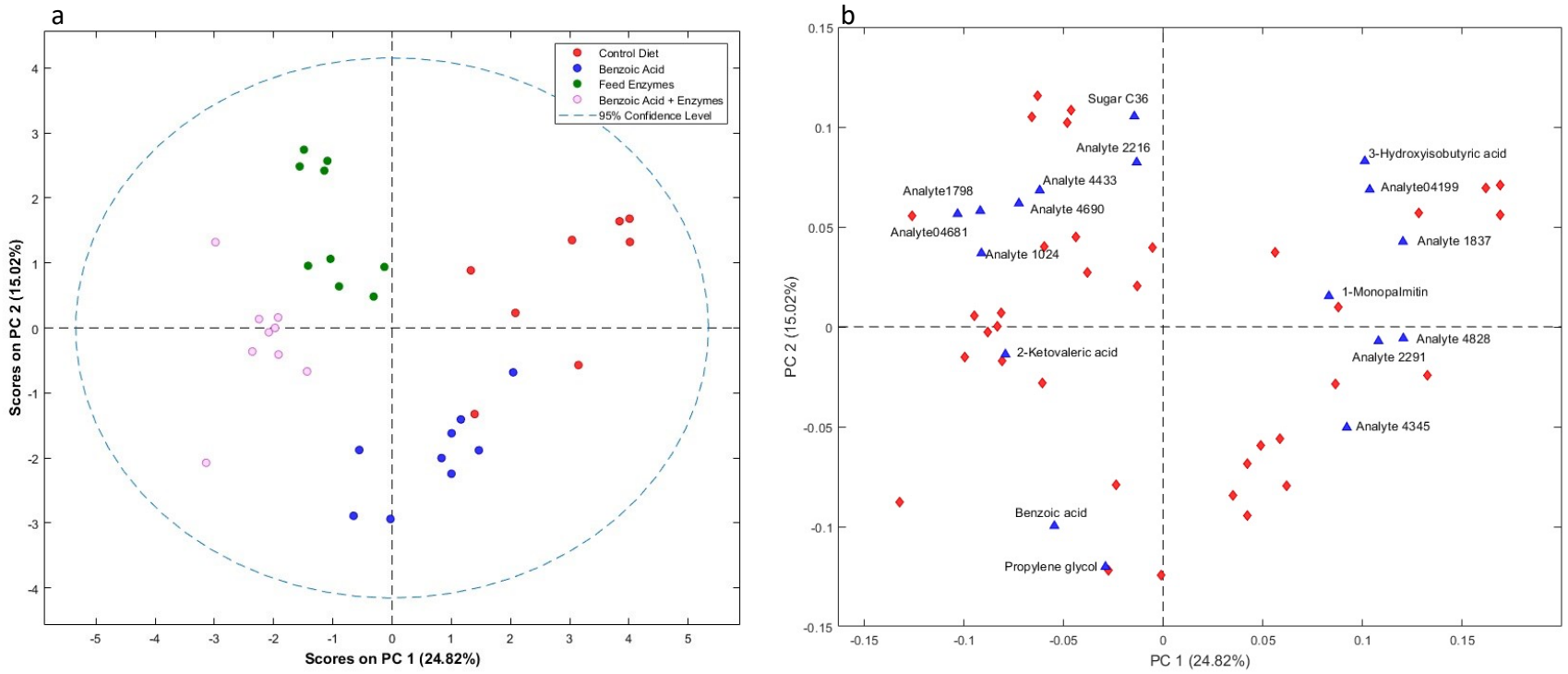

Figure 3.5. PCA results of 17 metabolites selected by two-step hybrid backward elimination/forward selection. These 17 metabolites could be used to identify differences in digesta from the cecum of pigs fed different diets. a. PCA score plot b. PCA biplot.

Analysis of individual metabolite differences by one-way ANOVA identified benzoic acid, phosphate and one unidentified analyte as significantly different between diets (Figure 3.6). Phosphate was significantly decreased in diets containing feed enzymes (FDR adjusted $P = 0.03$). Benzoic acid was detected as increased both diets containing added benzoic acid (FDR adjusted $P = 0.03$).

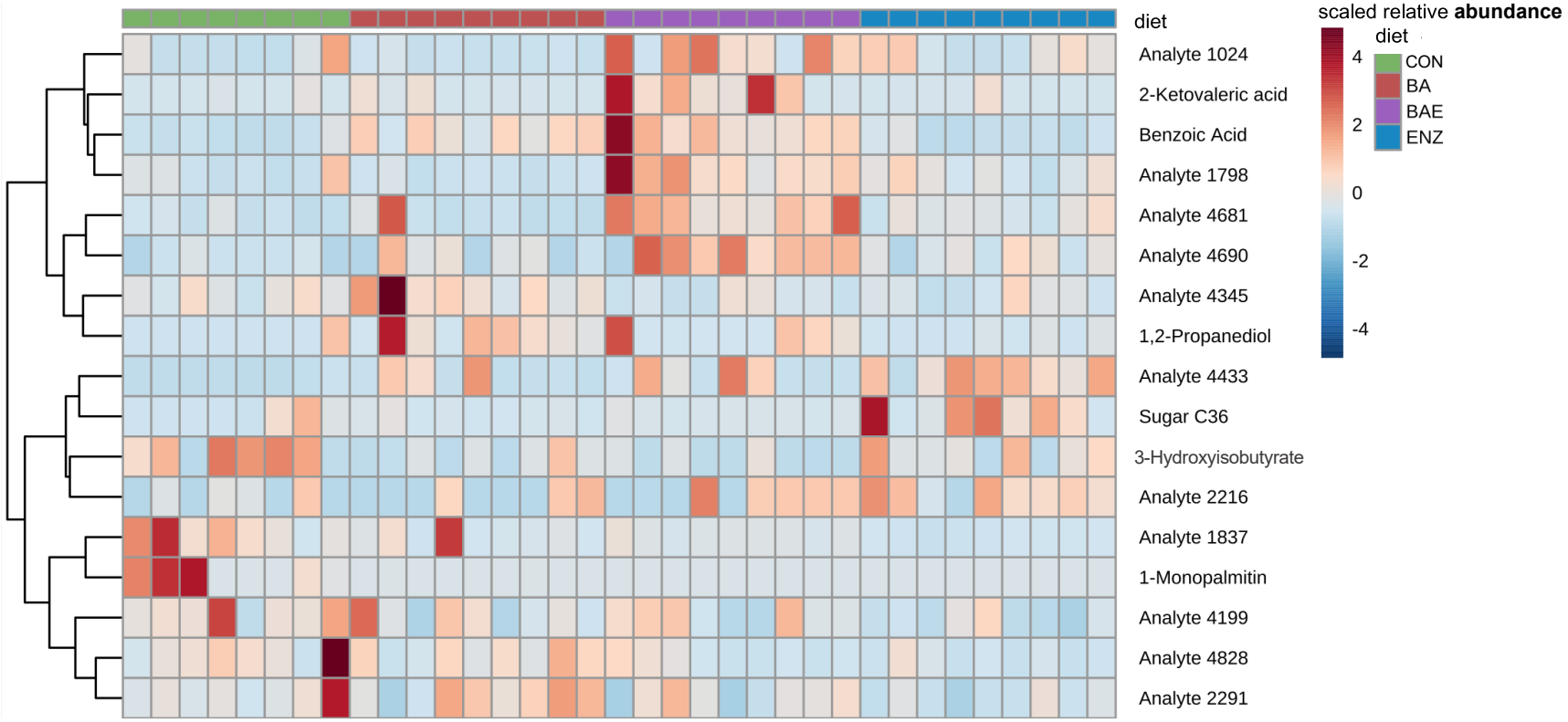

Figure 3.6. Heat map of metabolites identified during two-step FS on all four diets. Metabolites driving separation between cecal digesta of pigs fed one of four different diets including a control diet (CON), with added benzoic acid (BA), enzymes (ENZ) or both (BAE) were range-scaled and heatmaps were generated using Metaboanalyst 4.0 in R. Colour scale was generated using the Z-scores for each row.

Two-step feature selection identified differentiating metabolites in the cecum of each treatment compared to the control diet (Supplementary Table A1 and A2). Along with increased benzoic acid, the microbial metabolite 1,2 propanediol was higher in diets containing benzoic acid than the control diet (Figure 3.7). The addition of feed enzymes resulted in decreased abundance of fucose derived from plant polysaccharides, as well as decreased glycine, phenylalanine, and 1-heptadecanol (Figure 3.8).

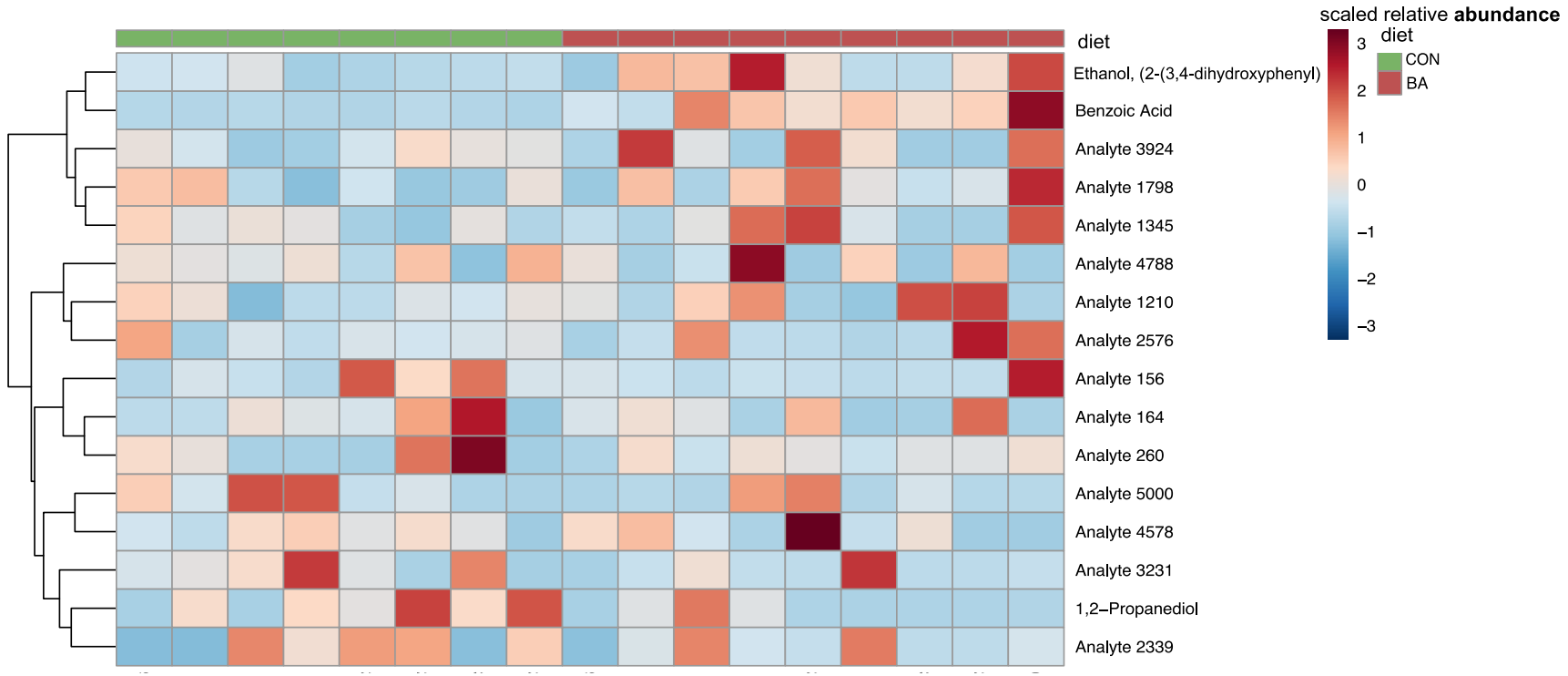

Figure 3.7. Metabolites identified as driving separation in cecal digesta of pigs fed either a control diet (CON) or with added benzoic acid (BA) were identified using a two-step FS. Heatmaps were generated based on separation results using Metaboanalyst 4.0 in R. Colour scale was generated using the Z-scores for each row.

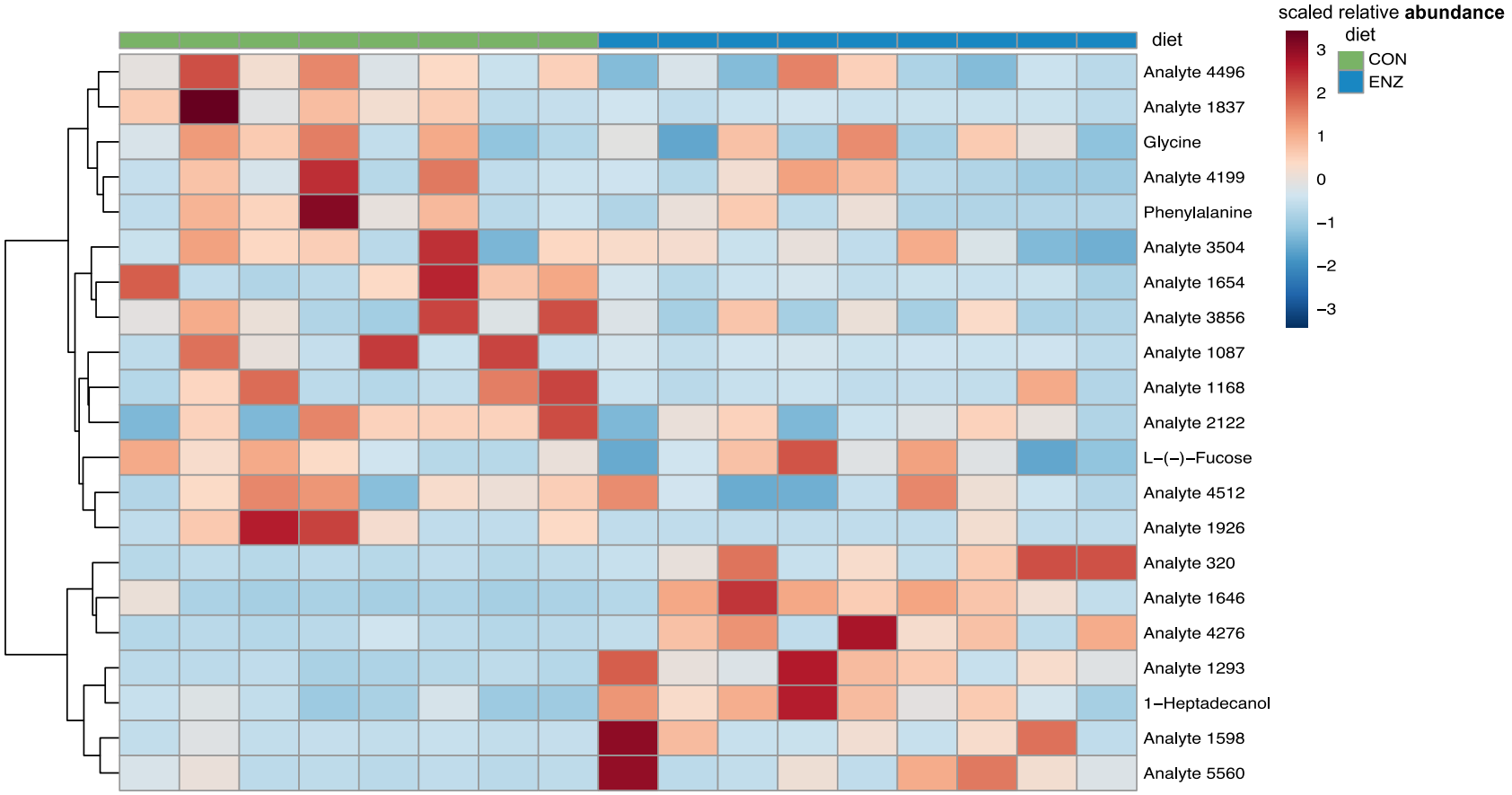

Figure 3.8. Metabolites identified as driving separation in cecal digesta of pigs fed either a control diet (CON) or a diet with added feed enzymes (ENZ) were identified using two-step FS. Heat maps were generated based on separation results using Metaboanalyst 4.0 in R. Colour scale was generated using the Z-scores for each row.

3.4 Discussion

In the present study, benzoic acid supplementation increased the presence of benzoic acid in digesta beyond the primary site of absorption. Within 24 hours of feeding, 60% of benzoic acid is absorbed in the small intestine by monocarboxylate transporter 1 (MCT1), where it is subsequently conjugated by the liver into hippurate and excreted in urine [33,34]. This novel finding indicates that at the evaluated dietary inclusion, this compound reaches the cecum in its undissociated form when supplemented. This indicates that more benzoic acid is being fed than can be absorbed by MCT1 transporters in the small intestine despite the efficient absorption previously reported [34]. Excess benzoic acid is conjugated with glycine into hippuric acid by the liver and excreted by the kidneys. No changes in glycine in the cecal digesta were detected for pigs fed benzoic acid which may indicate that no increased conjugation occurred in benzoic acid-fed pigs. However, it is not possible to ascertain what proportion of benzoic acid fed reached the large intestine within the scope of this study. The lack of feed efficiency effects demonstrated within this study could be due to the sensitivity of organic acids to barn hygiene [35]. Low pathogen load and intensive sanitation between batches of pigs is a proper swine management strategy but reduces the ability to detect differences in growth performance related to post-weaning diarrhea. Effective supplementation of organic acids may require consideration of dietary acid-base balance and fermentable carbohydrate level to adjust dietary levels of organic acids and achieve a change in digesta pH [35]. Further, the dose of BA approved for use in North America is sufficient for protecting against feed spoilage but may be insufficient to alter gut microbiota composition.

At the inclusion rates in the present study, feed enzymes had a more pronounced effect than benzoic acid on the abundance of individual taxa within the cecal microbiota as well as on microbial metabolism. Other studies have shown an effect of feed enzymes on *Lactobacillus*

species that is dependent on the cereal type included in the basal diets, where an effect of β-glucanase and xylanase has been shown on diets formulated with barley [36], as is the diet in our current study. Increased *Lactobacillus* abundance has been demonstrated alongside a decrease in fecal coliforms in response to a xylanase and β -glucanase complex [13]. This effect is likely due to β-glucan degradation, which is further demonstrated by the pronounced effects of oat inclusion on gut microbiota irrespective of enzyme feeding, when compared to inclusion of barley [36]. This is likely due to enzymes altering the availability of fermentable substrates within the diet in a manner that is dependent on the plant polysaccharide composition of individual cereal grains; consistent with the changes in the cecal microbiota observed in this study.

Taxa identified with increased relative abundance in the microbiota of enzyme-fed pigs included fibre fermenting families such as *Prevotellaceae* and members of order Bacteroidales. Key taxa found to be decreased in the cecum of pigs fed dietary enzymes include genera with known protein fermenting microbes such as *Selenomonas*, *Megasphaera*, and *Acidaminococcus* [37–39]. Decreased abundance of the taxa *Phascolarctobacterium* and *Oscillospiraceae*, which are known to decrease in diets with more fermentable carbohydrate was also observed (Sun et al., 2015). While it is not possible to differentiate the effects of individual enzymes within the scope of this study, previous studies have shown increases in the abundance of *Prevotellaceae* [15] and decreases in *Megasphaera* [17] in response to supplemental xylanase. This suggests that xylanase may play an important role in the results observed, likely through improving protein digestibility and degrading complex plant cell wall components into more easily fermentable compounds [19]. It is also notable that many of the taxa affected by dietary enzymes contain known propionate producers including *Veillonellaceae*, *Selenomonas*, *Megasphaera*, *Acidaminococcus*, and *Prevotellaceae* [39,41–43], however propionate levels are not different between the two groups,

suggesting that the increased abundance of *Prevotellaceae* in enzyme-containing diets is also responsible for a shift in primary propionate producing microbes away from those utilizing amino acids or lactate. Overall, this pattern suggests that improved digestibility of protein and alterations in fermentable plant polysaccharides in response to dietary enzymes alters cecal microbes producing propionate without a detrimental effect on propionate levels themselves.

Metabolomic results indicated that, in the presence of additives, changes in flow of dietary substrates may affect microbial metabolism in the cecum. When all four diets are examined together, metabolites such as 3-hydroxyisobutyrate indicate a change in gut inflammatory status. A product of microbial valine metabolism, 3-hydroxyisobutyrate has been identified as increased in inflammatory conditions affecting the gut such as celiac disease and ulcerative colitis [44,45]. This metabolite was found in greater abundance in the cecum of pigs fed control and enzyme diets than those fed benzoic acid diets. This suggests that less valine degradation takes place in the presence of benzoic acid in the cecum. This branched-chain amino acid metabolite is particularly interesting when considered alongside the increased 1,2-propanediol observed in pigs fed benzoic acid containing diets. A microbial cross-feeding product, 1,2-propanediol is generated from degradation of plant cell wall components such as fucose and rhamnose [46]. As branched-chain amino acid metabolism is a hallmark protein fermentation by microbes [47], this could be an important avenue for further investigation of the role of these additives in post-weaning diarrhea. This cross-feeding is an important function within the gut microbial community, supporting many beneficial microbes within the gut as well as production of propionate [43,48].

The production of short-chain fatty acids by the microbiota is also inhibitory to protein fermentation, and therefore may represent a beneficial impact of benzoic acid in the cecum [47]. Further, when the effects of ENZ are compared to the CON diet, we observe lower abundance of

glycine and phenylalanine in the cecum. This decreased abundance of amino acids reaching the hindgut, and therefore available for subsequent degradation by the microbiota in ENZ containing diets, may also be important to diarrhea outcomes; as is the decreased abundance of 1-heptadecanol. Previous studies have shown a correlation between 1-heptadecanol and Proteobacteria and a decrease in 1-heptadecanol following the introduction of a combination of live *Bifidobacterium*, *Lactobacillus, Enterococcus,* and *Bacilli* in a model of dysbiosis and immunosuppression [49]. The decreased abundance of 1-heptadecanol in the presence of feed enzymes along with a lower abundance of the plant oligosaccharide fucose suggest that enzymes support the development of fermentative capacity in the microbiota of pigs following weaning. With increased abundance of fermentable oligosaccharides as a microbial energy source, these plant cell wall components may be more readily degraded by the microbiota of ENZ pigs and support fibre fermenting genera such as *Prevotellaceae,* which is an important component of the weaned pig microbiota [50].

These differences in the presence of fibre fermenting genera and the production of metabolites from polysaccharide degradation support a role of feed additives in supporting the establishment of microbial metabolic pathways and species needed to degrade complex dietary components in the weaned pig. This could contribute to improved resolution of post-weaning diarrhea as fibre-fermenting species establish and expand within the intestine and exclude pathogens [2]. In conclusion, this demonstrates an effect of dietary additives on altering cecal microbial composition and metabolism during the sensitive period following weaning.

3. 5 References

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Chapter 4: A blend of medium-chain fatty acids, butyrate, organic acids, and a phenolic compound accelerates microbial maturation in newly weaned piglets

4.1 Introduction

Medium-chain fatty acids (MCFAs), phenolic compounds, and organic acids are commonly used in dietary additives blends during the weaning transition in pigs; their effects on the gut microbiota are thought to reduce the risk of gastrointestinal pathogen colonization and adverse outcomes during this vulnerable period in pig production [1,2]. Medium-chain fatty acids exert antimicrobial effects through disruption of phospholipid membranes [3], and have been shown to suppress gastric and intestinal pathogens including *Escherichia coli* and *Clostridium perfringens* [3–5]. Short-chain organic acids are often used following weaning to reduce gut pH and pathogen colonization in the gastrointestinal tract [6,7]. Phenolic compounds have also been shown to control pathogen colonization and improve gut barrier function in the intestine while increasing the abundance of many core constituents of the microbiota [2].

Synergistic effects of blends of MCFAs and short-chain organic acids have also been demonstrated compared to MCFAs or organic acids fed alone [8]. Previous studies have shown a positive effect of a blend of both MCFAs and organic acids on commensal ileal microbes during pathogen challenge [9], as well as on diarrhea, immune function, and fecal microbiota under non-challenge conditions [10]. Inclusion of these blends in weanling pig diets have also been shown to improve growth, feed efficiency, and nutrient digestibility [11,12].

While there are many studies demonstrating the synergistic effects of these ingredients in weanling pig diets, the mechanisms by which they affect microbial and host metabolism, and confer these benefits, remain to be examined. To gain an increased understanding of how a blend

of MCFAs, phenolic compounds, butyrate, and organic acids (MCOA) impact microbial and host metabolism, we performed an in-depth characterization of the metabolites in two intestinal sites, portal plasma, and systemic plasma at multiple time points through the weaning transition. These results were combined with microbiota sequencing analysis from the same intestinal sites to explore how modulation of microbiota altered metabolite pools compared to pigs fed a control diet. This approach provided novel insights into the possible mechanisms through which MCOA improve performance outcomes.

4.2 Materials and Methods

The study was approved by the Dutch Animal Ethics Committee (AVD2040020184665) and carried out at the Swine Research Centre of Trouw Nutrition R&D (Sint Anthonis, the Netherlands).

Animal Performance Data

At weaning (22-25 days of age with average body weight 7.7 kg ± 0.7), a total of 108 pigs (Hypor Maxter × Hypor Libra) were selected from 20 litters of origin and assigned to one of two treatment groups (Table 4.1), those receiving the control diet (CON) and those receiving a control diet with the addition of a blend of medium chain fatty acids, organic acids, slow release C12, target release butyrate and a phenolic compound (MCOA) (Presan FX, Trouw Nutrition, Amersfoort, NL) supplemented at 0.2% on top of the control diet. Diets were isoenergetic and isonitrogenous (Table 4.2). Groups were balanced for body weight, colostrum intake, and sex. Pigs were housed in one of six pens with 18 pigs/pen according to treatment and individual feed and water intake data were collected using electronic feeding and water stations (Schauer Agrotronic, GmbH, Austria). Body weights were collected at 2 days prior to weaning, and on days 0, 2, 4, 6, 13 and 27 post-weaning as well as at time of euthanasia. Growth performance data were compared

using the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC) with treatment as a fixed effect and pen as a random effect.

Table 4.1. Diet formulation of basal diet.

Ingredient	%[1]
Barley	12.3
Wheat	35.0
Maize	10.2
Wheat bran	2.0
Soybean meal 48	20.0
Potato protein	2.5
DL-Methionine 99%	0.18
L-Lysine HCl 98%	0.51
L-Threonine 98%	0.16
L-Tryptophan 98%	0.03
L-Valine 96.5%	0.08
Na Bicarbonate	0.26
Ca Carbonate	0.73
Monocalcium phosphate	0.75
Salt (NaCl)	0.44
Premix	1.0
Lactose	6.5
Sugar	2.5
Soybean oil	4.7
Vitamins and other	0.2

[1] Ingredients listed constituted the control diet, while the MCOA diet was identical except for the addition of 0.2% MCOA (Presan FX, Trouw Nutrition, Amersfoort, NL) on top.

Table 4.2. Nutrient analysis of experimental diets.

Nutrient[1]	CON	MCOA
Net Energy, kCal/kg[2]	2550	2550
Moisture %	10.7	10.7
Crude protein, %	19	19.1
Crude fat, %	6.4	6.5
Crude fibre, %	2.7	2.7
Ash, %	4.6	4.6
Na, %	0.25	0.26
K, %	0.75	0.76
Mg, %	0.2	0.21
Ca, %	0.67	0.67
P, %	0.45	0.46
Zn, mg/kg	117	108
Sorbic acid, mg/kg[3]	<1	130

[1] Diets were formulated using a wheat, barley, maize, and soybean meal base to meet or exceed all nutrient requirements. Nutrient levels were determined using standard analytical methods. Moisture was determined according to method 930.15 (AOAC, 2007) and nitrogen was determined by the combustion method (method 990.03; LECO FP 528 MI, USA) using the LECO Nitrogen analyzer and crude protein calculated as nitrogen × 6.25. The crude fat was determined as an extraction method 920.39 according to AOAC (AOAC, 2007). The ash content of the diets was measured according to method 942.05 (AOAC, 2007). Mineral analyses were completed according to NEN-EN 11510(EN) specifications (2017). This method uses the inductively coupled plasma atomic emission spectroscopy (ICP-AES) method to determine minerals in animal feed after dry ashing. Sorbic acid was determined using the method described by Canale et al. (1984) .

[2] Net Energy calculated using Bestmix® (Adifo N.V., Maldegem, Belgium) formulation software.

[3] Analysis of sorbic acid content was performed in order to confirm the addition of MCOA

Sample Collection

On the day prior to weaning, 10 piglets were selected for sample collection based on body weight, colostrum intake, and sex. This group was used as reference group that had not been exposed to weaning stress. On D 3, 5, 7, and 14 post-weaning, a subset of pigs was selected for sample collection based on representative feed intake for their treatment group and balanced for sex (n=10/group/day). Samples from CON animals only were also collected on D28 (n=10). Prior to euthanasia, jugular venipuncture was performed, and plasma collected for metabolomics analysis. Blood was stored on ice immediately upon collection, and plasma was isolated by centrifugation (10 minutes at 2000×G at 4°C) within 2 hours after blood collection. Following blood sampling, pigs were euthanized, and samples collected for microbiota sequencing and metabolomics analysis. First, portal vein blood samples were collected, and plasma isolated for metabolomics analysis following the same procedure as for jugular plasma. From the gastrointestinal tract, contents from the length of the small intestine were collected and homogenized, while in the large intestine, contents of the mid-colon were sampled. All samples were snap frozen on dry ice and stored at -80°C until further analysis.

Metabolomics

Plasma and digesta samples were processed by the Center for Proteomics and Metabolomics (Leiden University Medical Centre, Leiden, the Netherlands) and submitted to untargeted LC-MS/MS with a positive and negative ionization phase. Samples were first homogenized, and protein precipitation performed [13]. Metabolomic analysis was performed using an LC-MS/MS based SWATH method with a Shimadzu Nexera X2 system (Shimadzu, Hertogenbosch, The Netherlands). The MS was a Sciex TripleTOF 6600 (AB Sciex Netherlands B.V., Nieuwerkerk aan den IJssel, NL) operated in positive and negative ESI mode. Peaks were

identified using data independent acquisition (SWATH) using the public metabolite (VS12) database to align and identify metabolites [14].

Following peak identification, metabolite differences for each day and location were analyzed using MetaboAnalyst 5.0. Metabolite intensities were first normalized using quantile normalization and range scaling. Following processing, metabolome differences were visualized using PCA and ANOSIM analysis on Bray-Curtis distances performed to compare differences in metabolomes. Differences in individual metabolites were assessed using FDR adjusted t-tests where significance was determined as $P < 0.05$ and trends at $P < 0.10$. Overrepresentation analysis was performed on jugular metabolite samples using the list of significantly different metabolites to identify metabolic pathway differences between groups at each timepoint.

Microbiota Analysis

RNA extraction was performed on lysed cells (MagNA Lyser; Roche, Burges Hill, UK) using as the MO BIO RNA isolation kit (Carlsbad, CA, USA) without the use of β-mercaptoethanol and DNase in steps as previously described (15). Amplicon libraries of the V3-V4 region of 16S rRNA were constructed following the Illumina protocol with modifications to reduce PCR bias [15].

Sequencing was performed on the Illumina MiSeq platform with a read length of 300bp. Raw sequence data was processed and annotated in Mothur (v 1.39) where reads were merged, filtered for quality, and aligned using the SILVA (NR-123) database [16]. Aligned reads were further filtered to exclude chimeras and clustered into OTUs using VSEARCH with a cut-off of 97% identity [17]. Sequence data was uploaded to the NCBI sequence read archive (Project ID: PRJNA928417). Processed OTU tables and phylogenetic trees were then imported into R where the effects of MCOA on both alpha and beta diversity were analyzed on each day using the

phyloseq package [18]. Alpha diversity was examined using both Chao1 and Shannon indices and compared using a Wilcoxon ranked-sum test. Differences in beta diversity were compared using the adonis2 function (PERMANOVA) in the R package vegan on Bray-Curtis and UniFrac matrices. Differentially abundant taxa were examined using LEfSe analysis [19].

Due to the high number of low abundance and low incidence OTUs in the small intestine of pigs sampled on D3, 5 and 7, a successional core was identified from the microbiota of D14 pigs and used for comparisons of core microbiota assembly in the small intestine at earlier timepoints [20,21]. Due to the disruption of weaning, core microbes were defined as those present in > 60% of D14 individuals with > 5% relative abundance. These OTUs were then compared between groups at D7 using a Wilcoxon ranked-sum test. To further assess the relationship between metabolites and core microbes in the small intestine, regularised Canonical Correlation Analysis (rCCA) was performed using a cross-validation (ridge) method for regularisation in the R package mixOmics [22,23]. Results were visualised using a relevance network plot with a correlation threshold of 0.6 and a cluster image map showing all correlated relationships between microbes and metabolites identified using rCCA [24].

4.3 Results

Pig Performance

Average daily gain (ADG) from D0-13 (CON n = 19, MCOA n = 22) was higher for pigs fed MCOA (111 vs 71 g/d with $P = 0.04$; Figure 4.1), this growth rate is lower than expected for pigs of this age in this facility; likely due to the increased amount of handling required for this experimental protocol. Feed efficiency was not significantly different between groups.

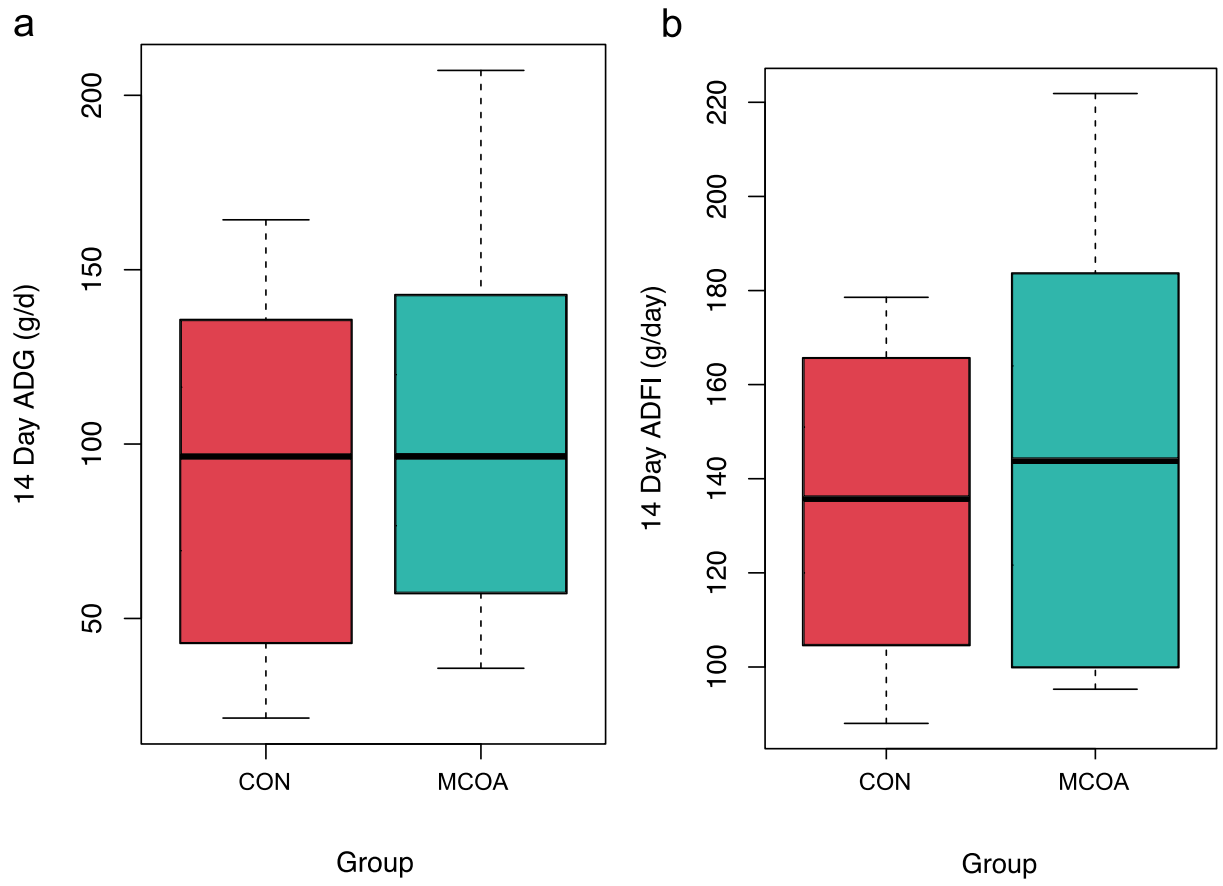

Figure 4.1. Comparison of growth performance. a. Average daily gain (ADG) and b. average daily feed intake (ADFI) for pigs fed a control diet (CON) or a control diet supplemented with blend of medium chain fatty acids and organic acids on top (MCOA) for the first thirteen days post-weaning. ADG was significantly different (P = 0.04) while ADFI was not significantly different (P = 0.19) between groups (n=19 for CON and 22 for MCOA) for the thirteen-day period.

Metabolome

An overall shift in the global metabolite pool was observed at all time points, with the most noticeable difference at D7. Although feed intake was relatively low at D3 and 5 post-weaning in both diets (Supplemental Figure A1), analysis of similarity on Bray-Curtis distances showed a small but significant difference between plasma metabolome on both days (P = 0.004, R^2 = 0.29 and P = 0.007, R^2 = 0.29 respectively; Figure 4.2). No significant differences were detected in

intestinal metabolites at D3 or 5. A more pronounced separation, with a greater number of significantly different metabolites was observed at D7 and 14 in jugular plasma ($P = 0.001$, $R^2 = 0.82$ and $P = 0.001$, $R^2 = 0.70$ respectively, Figure 4.2). When individual metabolites were compared, key metabolite groups with noted differences between MCOA and CON animals included markers of tissue deposition and fatty acid metabolism (Figure 4.3), bile acid components (Figure 4.4), tryptophan metabolites (Figure 4.5), and B vitamins (Supplemental Figure A2). Metabolic pathway results showed two significantly overrepresented pathways in MCOA pigs for tryptophan metabolism and riboflavin metabolism. In CON animals, three significantly overrepresented pathways for purine metabolism, betaine metabolism, and oxidation of branched-chain fatty acids were detected (Supplemental Figure A3). A trend for enriched beta oxidation of very long chain fatty acids and pyrimidine metabolism were also identified.

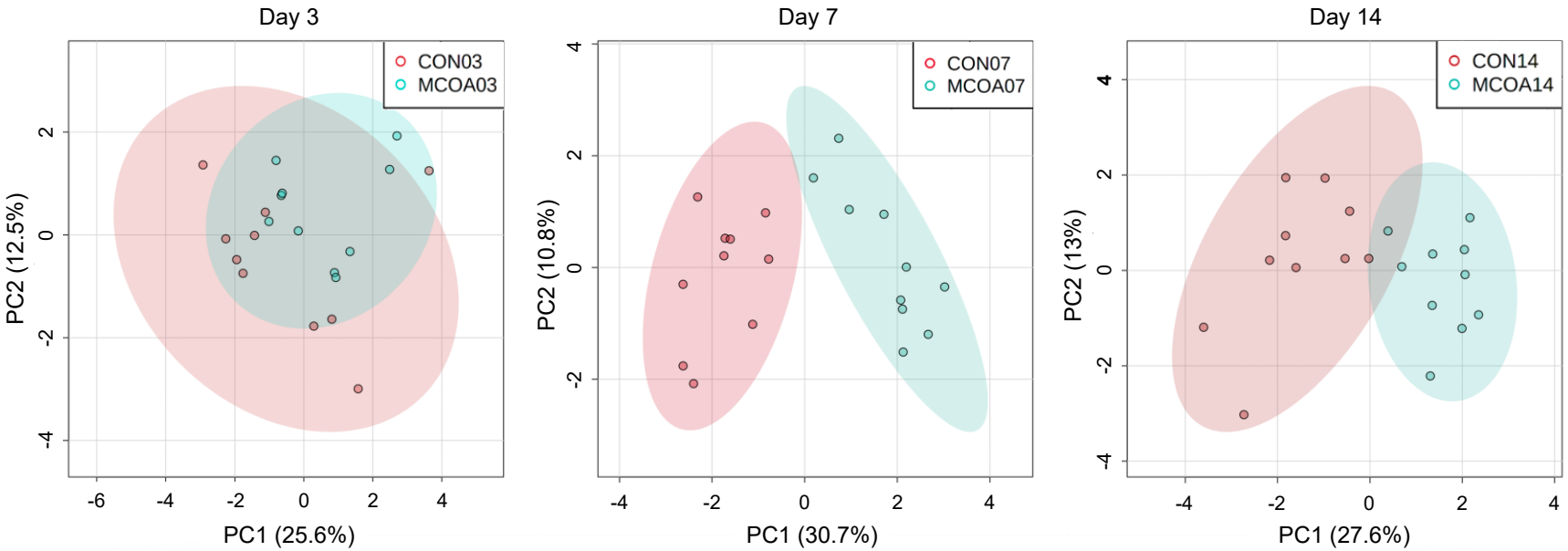

Figure 4.2. Summary of overall effects of MCOA on weaned pig metabolome. PCAs of metabolites on D3, 7, and 14 in jugular plasma; Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14).

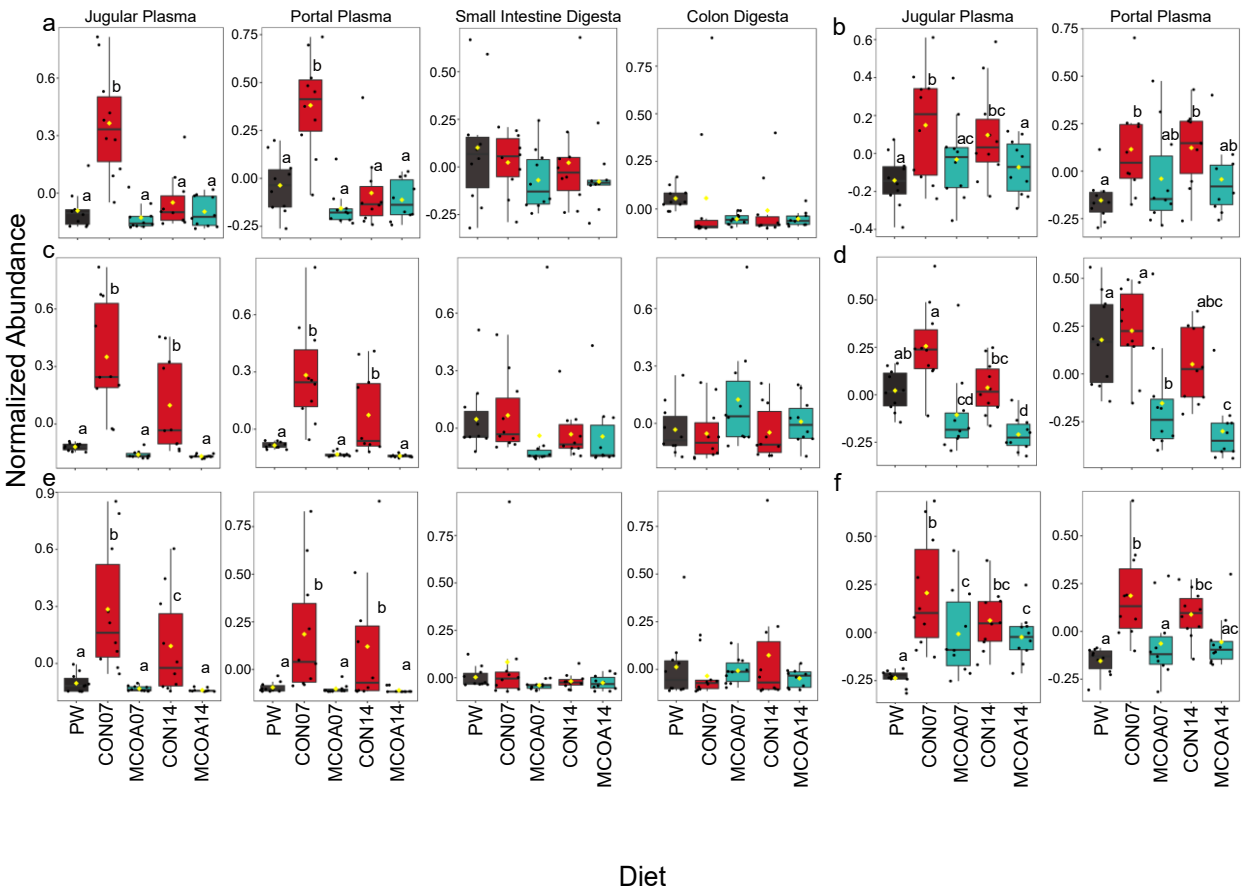

Fig 4.3. Whole-body metabolic indicators of tissue deposition. Differences between pre-weaning, MCOA, and control groups shown on D7 and 14 include a. Creatine; b. Creatinine; c. Acetylcarnitine; d. L-carnitine; e. β-Hydroxybutyrate; f. Homocysteine. Metabolomics was performed using LC-MS/MS with a SWATH method for peak identification and analyzed in Metaboanalyst 5.0. Compounds shown in figures 2b, d and f were not captured in intestinal LC-MS/MS. Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Pre-weaning samples are denoted as PW. Groups with statistically different means ($P < 0.05$) are denoted by different letters.

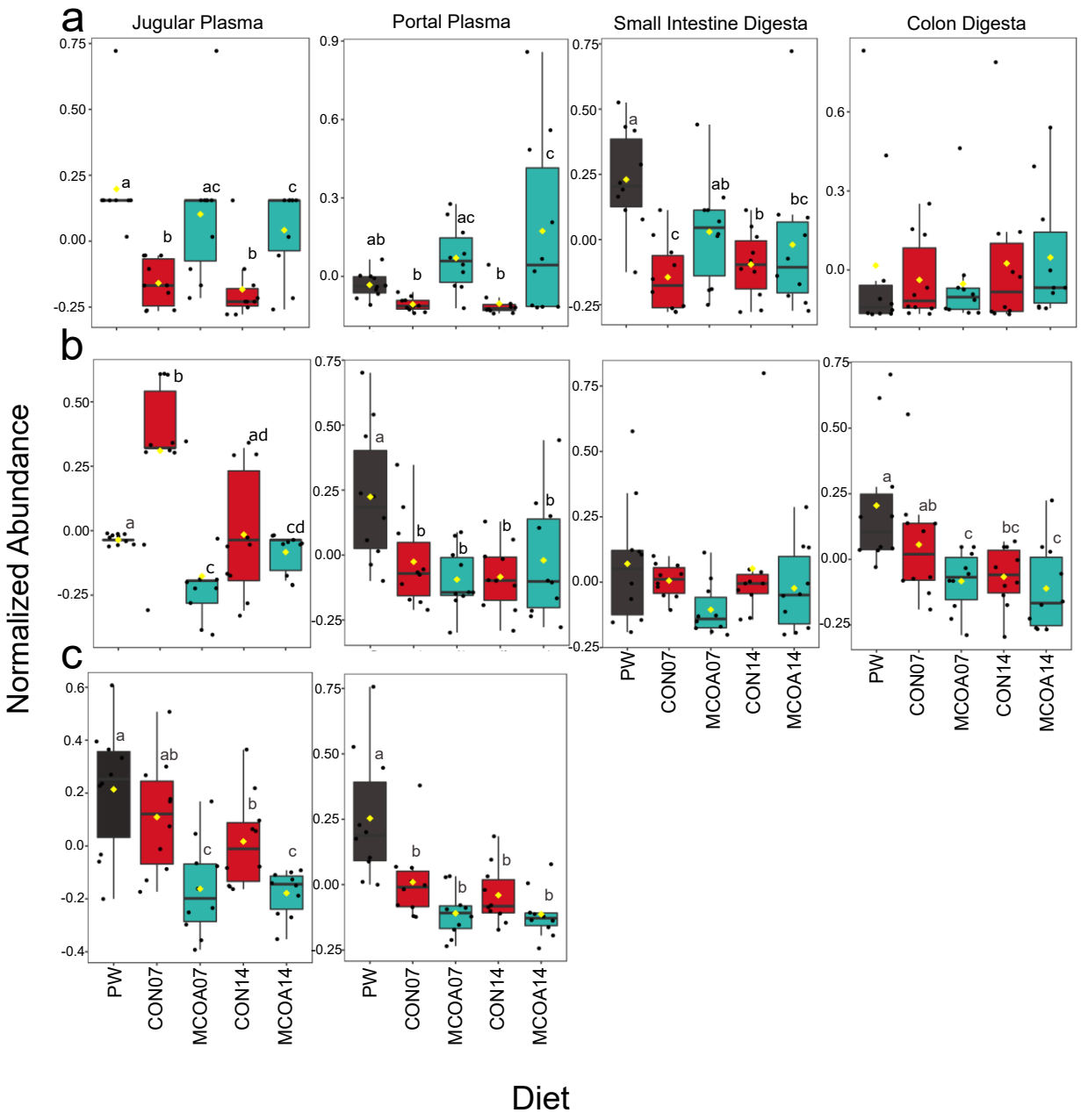

Figure 4.4. Bile-associated metabolite differences observed on D7 and 14 post-weaning in response to feeding a control diet or a diet containing MCOA. a. Cholic acid; b. Choline; c. Taurine. Metabolomics was performed using LC-MS/MS with a SWATH method for peak identification and analyzed in Metaboanalyst 5.0. Taurine was not detected intestinal metabolome. Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Pre-weaning samples are denoted as PW. Groups with statistically different means ($P < 0.05$) are denoted by different letters.

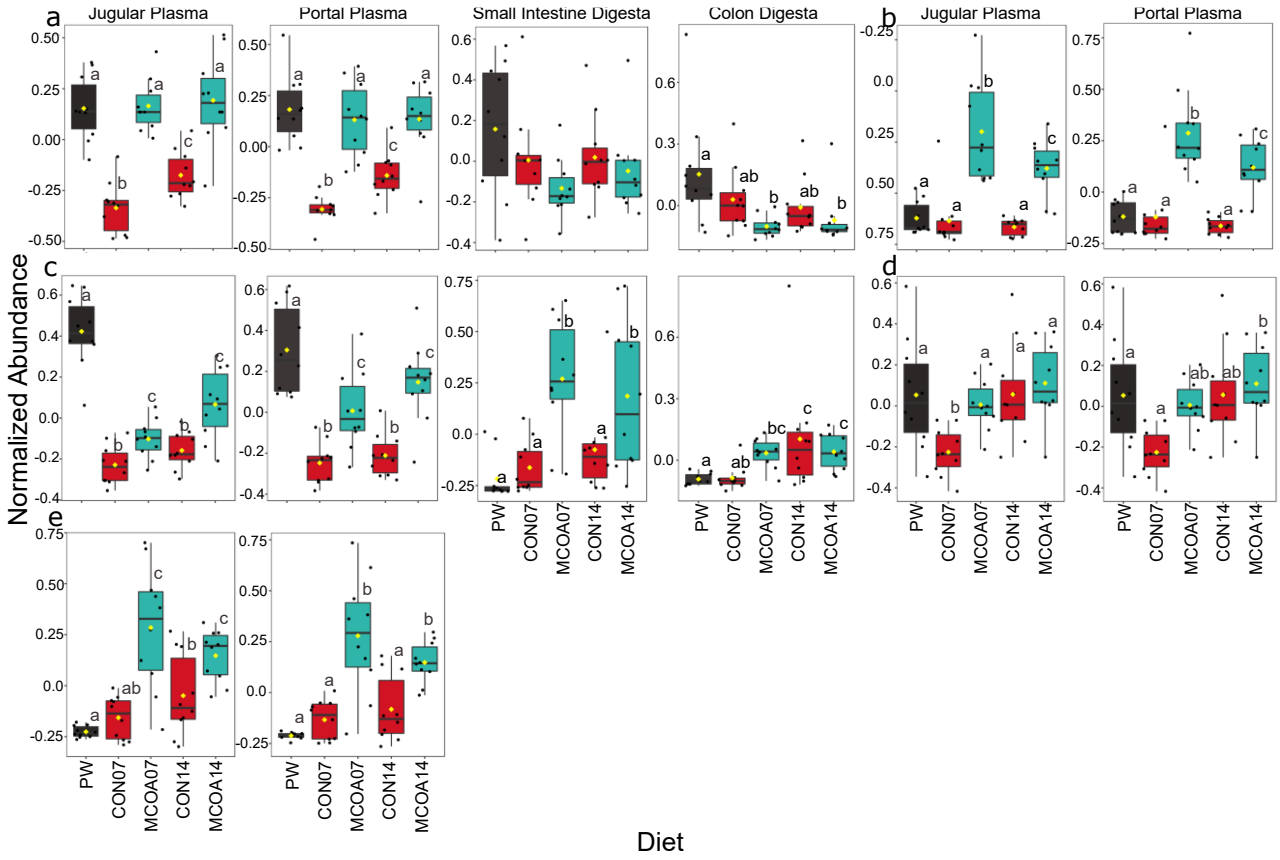

Figure 4.5. Tryptophan and metabolite alterations in response to a control diet or a diet containing MCOA. a Tryptophan; b. Indole-3-carboxylic acid; c. N-Acetyltryptophan; d. Indole-3-carboxyaldehyde; e. 3-Indolepropionic acid. Metabolomics analysis was performed using LC-MS/MS and a SWATH peak identification method. Statistical analysis was performed in Metaboanalyst 5.0. Compounds shown in 4b, d and e were not identified in intestinal metabolomics. Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Pre-weaning samples are denoted as PW. Groups with statistically different means ($P < 0.05$) are denoted by different letters.

Differences in small intestinal metabolome were only detected on D7 post-weaning (P = 0.002, r^2 = 0.27). In the colon, small but significant global differences were detected on D3 (P = 0.04, r^2 = 0.10), while more pronounced changes were found on D7 and 14 (P = 0.001, r^2 = 0.55 and P = 0.001, r^2 = 0.30 respectively). The global differences observed in intestinal metabolome were not due to the residual presence of additive components in the digesta at either location. Differences in individual gastrointestinal metabolites, measured through FDR-adjusted t-tests, were only observed at D7.

Intestinal Microbiota

Analysis of relative abundance data from the small intestine at all time points revealed numerous low abundance opportunistic taxa in the small intestinal microbiota at D3, 5 and 7, which limited statistical comparisons of important native microbiota constituents at early timepoints. To overcome this interpretation challenge in the early post-weaning period, a successional core of microbes was identified using D14 sequencing results. The abundance of these core taxa was then examined at earlier timepoints to measure microbial succession during this period. At D14, a total of four taxa comprising 83-100% of the small intestinal microbiota were identified as core genera: *Clostridium sensu*; Escherichia_Shigella, *Lactobacillus* and *Streptococcus*. Comparison of these taxa between MCOA and CON fed animals revealed a trend toward increased *Lactobacillus* relative abundance at D7 post-weaning in MCOA fed pigs (P = 0.05) (Figure 4.6d). This re-establishment of *Lactobacillus* is shown in the small intestinal microbiota of MCOA but not CON fed pigs at D7 and coincides with the time point where the most pronounced differences in metabolome in all compartments was observed.

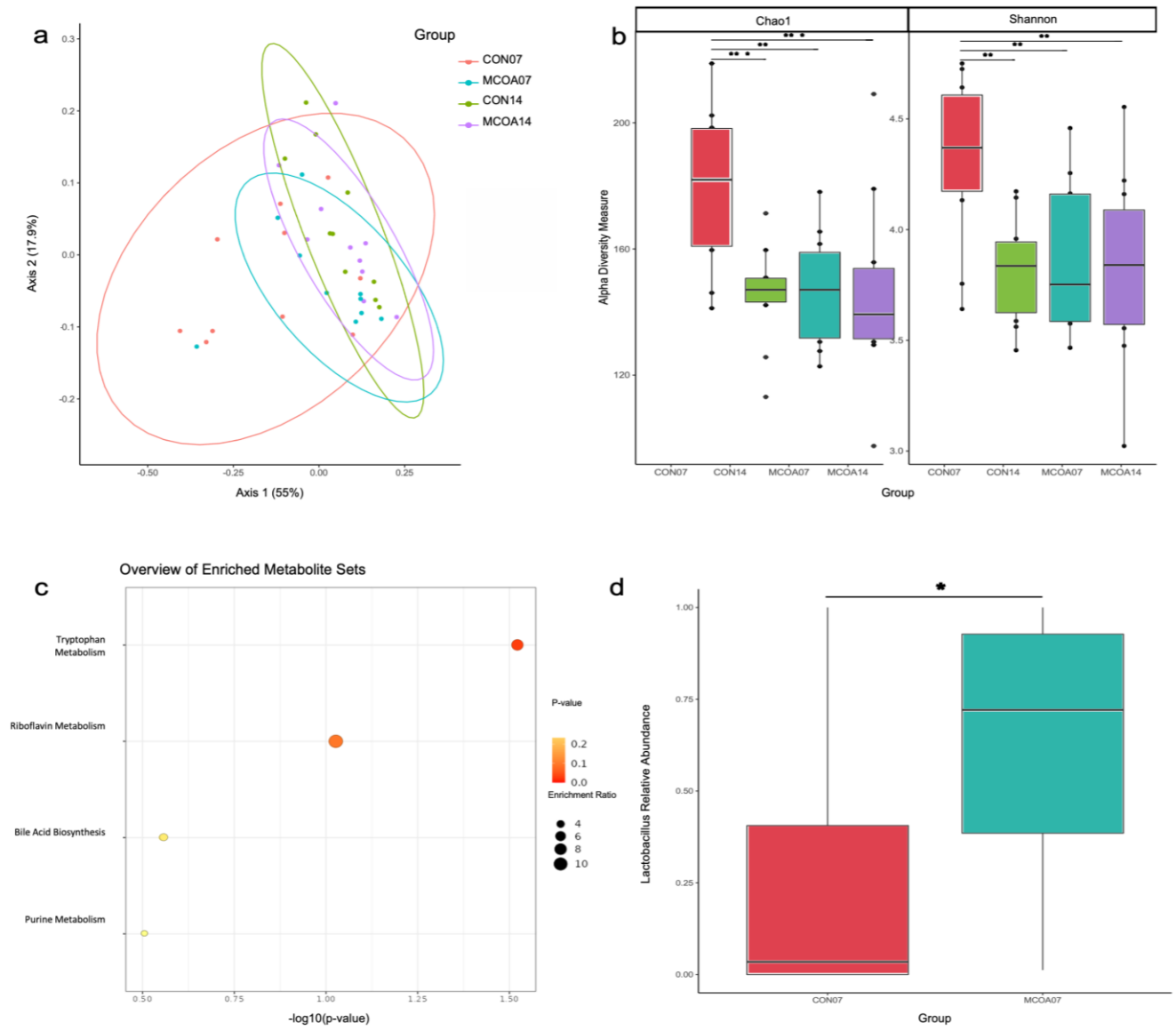

Figure 4.6. Summary of overall effects of MCOA on weaned pig microbiota and metabolic pathways. a. Weighted UniFrac distance of colon microbiota on D7 and 14; b. Alpha diversity measures of colon microbiota on D7 and 14; c.Metabolic pathways enriched in the jugular plasma of MCOA pigs on D7 post-weaning; d. *Lactobacillus* relative abundance D7 in the small intestine. Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Differences are denoted as * $P < 0.10$, ** $P < 0.05$ and *** $P < 0.01$.

Analysis of colon microbiota showed differences in composition and alpha diversity metrics between treatment groups at D7 post-weaning. The microbiota of MCOA fed pigs at D7 tended to be different than that of CON fed pigs (P = 0.09), and more closely resembled the microbiota of pigs at D14 post-weaning in measures of both overall abundance and composition (Figure 4.6a and 4.6b). The variability of individual microbiota composition, measured by homogeneity of dispersion, in CON fed animals at D7 was quite high (Figure 4.6a) compared to those at D14 (CON07 vs. CON14, P = 0.001), while that of MCOA fed animals was not significantly different from D14 animals (MCOA07 vs. MCOA14, P = 0.13).

Microbiota and Metabolome Networks

Network analysis using the R package mixOmics [24] revealed a positive correlation between *Lactobacillus* and abundance of tryptophan ($P < 0.05$, $r^2 = 0.61$) and tryptophan metabolites ($P < 0.05$, $r^2 = 0.40 - 0.62$) found in jugular plasma at D7 post-weaning (Figure 4.7a, 4.7b).

As *Lactobacillus* species have bile-tolerance mechanisms [25], the relationship between cholic acid and *Lactobacillus*, both of which are more abundant in MCOA fed pigs at D7, was explored in pigs sampled at all timepoints using Spearman correlation. A trend for a positive correlation was observed (P = 0.08, rho = 0.21; Figure 4.7c).

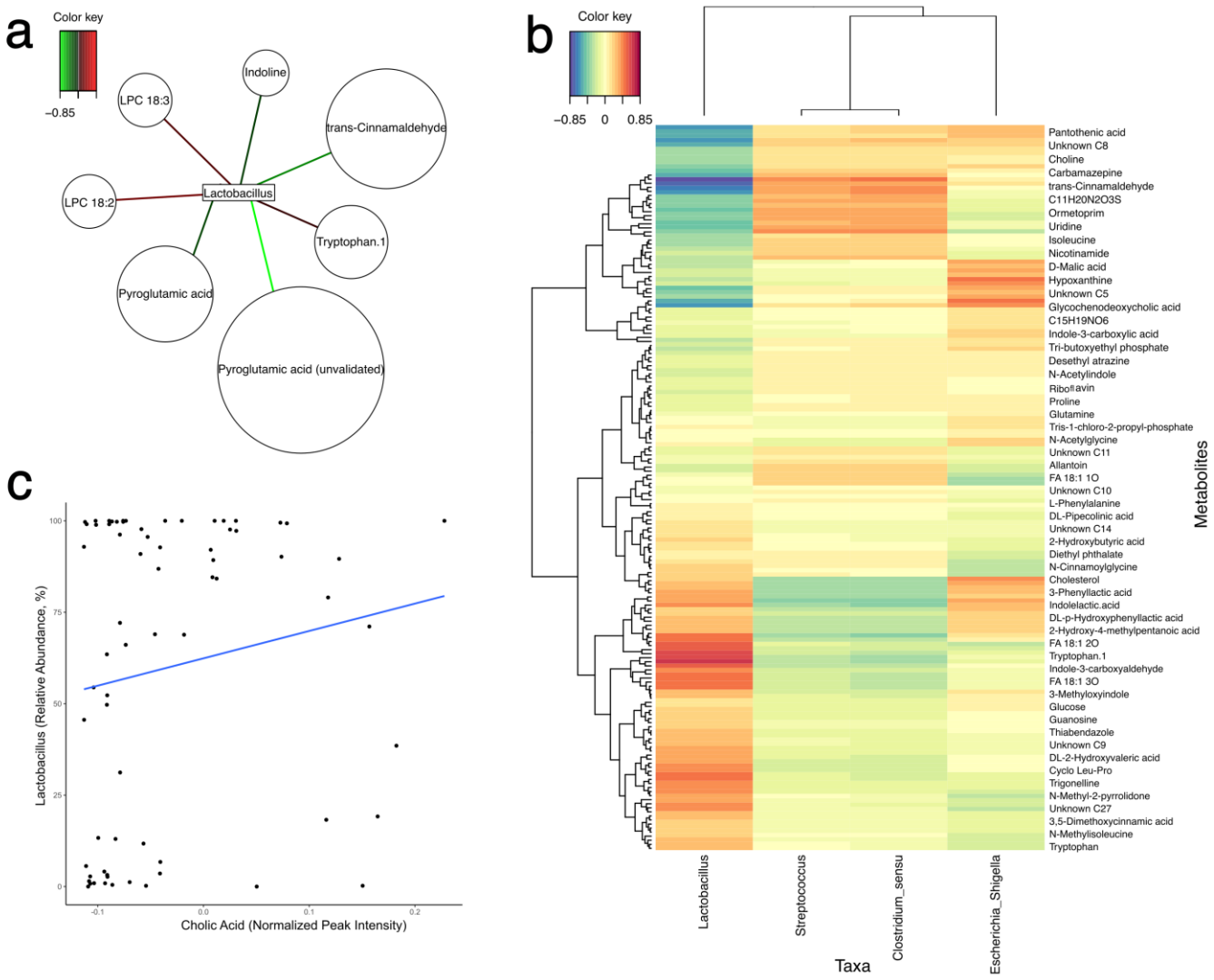

Figure 4.7. Microbe-metabolite relationships. a. Network analysis of core SI intestinal microbiota and metabolites at D7 with a significant correlation ($P < 0.05$) threshold r^2 of 0.6; b. Microbe-metabolite network analysis results for all significant correlations ($P < 0.05$) with core small intestine microbial taxa on D7; c. Correlation between *Lactobacillus* relative abundance and cholic acid in all pigs from the study ($P = 0.08$, rho = 0.21).

4.4 Discussion

Patterns of metabolome differences

The overall pattern of metabolite changes, with the strongest difference between metabolomes observed at D7 post-weaning, is particularly relevant given that it coincides with a time when the risk of *E. coli* associated post-weaning diarrhea is high and impacts of post-weaning interventions may be important for preventing diarrheal disease [26]. This is also a time where the initial acute stressors of weaning are resolved, and feed intake begins to rebound [26]. Higher feed intake at D7 and 14 would result in higher MCOA intake, contributing to the global differences observed on these days as well as to the potential mechanism underlying the global difference in intestinal metabolome observed at D7.

The fact that differences observed in the intestinal metabolite profiles did not include the additive components themselves is not unexpected as previous studies in weanling pigs have shown most MCFA digestion and absorption occurs prior to the end of the proximal small intestine, and abundance of these compounds, except for slow-release C12, drops sharply in subsequent sections of the GI tract [5,27]. Consistent with our results, it is expected that these compounds would not be observed in total small intestine or colon contents. However, this result alongside the changes in individual metabolites observed demonstrate the role of MCOA in altering metabolic networks beyond the metabolism of additive residues.

Metabolites reflective of impacts on tissue deposition

Several notable differences in metabolic markers of fatty acid metabolism and tissue deposition were detected in jugular plasma at D7 and 14 post-weaning. The prevailing pattern suggests improved energy balance and muscle metabolism alongside decreased fatty acid catabolism in MCOA fed pigs. Key evidence supporting this is the pattern of decreased creatine,

creatinine and β-hydroxybutyrate observed in MCOA fed pigs at these time points (Figure 4.3a, b and e). At D7 post-weaning, decreased acetylcarnitine, L-carnitine, and homocysteine also support this finding (Figure 4.3c, d and f). Except for homocysteine and creatine, these differences were also observed on D14 indicating ongoing effects of MCOA in increasing tissue metabolism post-weaning. Carnitine abundance is an important indicator of fatty acid metabolism and for overall metabolic status due to its effects on lipid and glucose homeostasis [28,29]. L-carnitine levels are tightly regulated in response to whole-body nutrient sensing, matching the amount of fatty acid metabolism to what is needed to regulate metabolic status [29]. The lower levels of L-carnitine in MCOA fed pigs may indicate less tissue catabolism at D7 and 14 post-weaning. At the same timepoints, lower circulating creatinine observed in MCOA fed pigs (Figure 4.3a), also suggests higher muscle metabolism. This is further supported by the higher growth observed from D0-13 of pigs fed MCOA. There was a dramatic increase in creatine post-weaning in CON fed pigs, consistent with reduced feed intake alongside a corresponding reduction in metabolic activity [30], while MCOA fed pigs did not exhibit this increase. Lower levels of creatinine (Figure 4.3b) are also seen in MCOA fed pigs, while CON fed pigs display elevated creatinine on D7 and 14 further indicating decreased metabolic activity and negative energy balance in CON fed pigs post-weaning [31,32]. This is further supported by significant overrepresentation of metabolites in pathways for oxidation of fatty acids (Supplemental Figure A3). Higher levels of riboflavin observed in plasma at D7 and 14 in MCOA fed pigs (Supplemental Figure A2) may also be related to these changes in energy balance. Levels of the B-vitamins niacin, riboflavin, and folate may also reflect creatine synthesis as they provide required methyl groups [33]. During fasting, methyl requirements for the methionine cycle must be met through remethylation of homocysteine, which depends on B-vitamin availability [34]. This results in an inverse relationship between homocysteine, creatinine

and B-vitamin metabolism and status [35], as observed here. At D7 post-weaning, riboflavin metabolites were found to be significantly enriched in jugular plasma of MCOA fed pigs (Figure 4.6c), while nicotinamide and pantothenic acid did not show the same pattern (Supplemental Figure A2). β-hydroxybutyrate was also elevated in CON fed pigs at D7 and 14 post-weaning (Figure 4.3e), indicating ongoing negative energy balance in CON fed pigs [36], despite comparable feed intake between groups (Figure 4.1b). The differences in these metabolites taken together illustrate decreased body fat mobilization in MCOA fed pigs at D7 and 14 post-weaning, which could in part be due to improved nutrient digestion, absorption, and subsequent alterations in nutrient sensing in the gastrointestinal tract in the presence of MCOA [37].

MCOA Supplementation alters bile components

At D7 post-weaning, MCOA fed pigs had increased abundance of cholic acid in the portal plasma, jugular plasma, and colon, suggesting an increase in bile acid production and secretion (Figure 4.4a). This change was accompanied by a decrease in the abundance of the bile acid precursor taurine in portal and jugular plasma (Figure 4.4b, c). Taurine also has an important role in osmoregulation [38], and may therefore be associated with post-weaning edema. Decreased plasma choline in MCOA pigs may also be due to changes in bile production and secretion, as it is the precursor to phosphatidylcholine, the primary phospholipid in bile as well as an important regulator of cholesterol homeostasis [39,40]. This alteration in primary bile metabolites persisted at D14 post-weaning, showing an effect of MCOA on bile metabolism throughout the sensitive post-weaning period as shown in Figure 4. Medium-chain fatty acids are known to increase the production of primary bile acids compared to other dietary sources of fat [41]. This could be particularly advantageous in the post-weaning period where bile acid and lipase production are

inadequate [42–44], and decreased feed intake may alter signaling for bile acid production, resulting in decreased nutrient digestibility [45,46].

MCOA supplementation increases generation of bioactive indoles

Alongside changes in bile metabolites, pigs fed MCOA displayed higher concentrations of tryptophan at D7 post-weaning in the portal and jugular plasma, while a lower concentration was detected in the colon (Figure 4.5a). At the same time-point, higher levels of five tryptophan metabolites – indole-3-carboxylic acid, 3-methyloxyindole, 2-methyloxyindole, indole-3-carboxaldehyde and 3-indolepropionic acid (IPA) were detected in the plasma of pigs receiving MCOA on D7 (Figure 4.5b, d, e; Supplemental Figure A4). Overrepresentation analysis further supported a significant increase in the tryptophan metabolism in MCOA-fed pigs on D7 post-weaning (Figure 4.4c). Increases in IPA, indole-3-carboxylic acid, 3-methyloxyindole, and 2-methyloxyindole were also observed in plasma at D14 (Figure 4.5b, d, e; Supplemental Figure A4). Indole and its derivatives can be absorbed via passive diffusion through cell membranes and readily move into portal circulation [47], and it is, therefore, not surprising that these metabolites did not differ in the digesta. Further, an increase in n-acetylated tryptophan, a microbial product [48], was detected in the small intestine of MCOA fed pigs (Figure 4.5c). These changes are indicative of an increase in tryptophan degradation by gastrointestinal microbes in MCOA fed pigs. As tryptophan metabolites are important signaling molecules [49,50], changes in tryptophan availability and production of bioactive molecules via microbial degradation are of particular interest to animal health outcomes. These tryptophan metabolites are commonly detected in a mature pig microbiota [51], and are therefore of interest for further investigation related to post-weaning microbiota assembly in this study.

Accumulating evidence shows that indoles are highly bioactive through aryl-hydrocarbon receptor (AhR) binding and are important regulators of gut barrier function with protective effects in models of irritable bowel disease [52–54]. Serum indole and IPA have previously been shown to be decreased in active colitis, while colonic tryptophan was elevated [51]. This supports a positive effect of MCOA on gastrointestinal tract function and inflammation as the opposite pattern is observed at a time where gastrointestinal inflammation is common [55,56]. Indole-3-carboxylic acid is also capable of binding AhR, though this has been less widely described [57]. Methylindole (skatole) is an intermediate in the production of indole-3-aldehyde from indole-3-acetate and is subsequently metabolized into 3-methyloxyindole in the liver via CYP450 [47]. Though not likely to be related to weaning outcomes, this metabolite may be of further interest for minimizing off-flavours in pork products.

Microbiota assembly differences in response to MCOA

Given the role of *Lactobacillus* spp. as important members of the porcine microbiota [58], which are long known to be sensitive to host stressors [59], their increased abundance in MCOA-fed pigs is an important indicator of microbiota re-assembly following weaning [60]. The positive correlation between this genus and cholic acid is of further interest, indicating that the inclusion of MCOA and resulting increase in cholic acid production may shape the small intestinal microbiota's re-assembly of *Lactobacillus* post-weaning (Figure 4.7c). Further supporting this is the consistent evidence in the literature of increased lactobacilli counts in a variety of age groups of pigs in response to a blend of dietary MCOA [11,12,61,62], suggesting that a protective effect of MCOA occurs through shaping a gut environment that allows an earlier *Lactobacillus* expansion post-weaning.

In the early post-weaning period, alterations in tryptophan and indole metabolites between MCOA and CON fed pigs suggests that MCOA may increase the assembly of microbes able to generate indole metabolites [63]. Production of IPA and the correlation with *Lactobacillus* abundance, may be of particular interest due to its beneficial effects on gut barrier function [52], requires microbial enzymatic machinery, and common commensal gut organisms *Lactobacillus, C. sporogenes* and *Peptostreptococci* possess the capability to generate IPA [47,63,64]. Encapsulated MCFAs have been shown to increase *Lactobacillus* spp. abundance in the stomach of pigs [5], supporting the possibility that tryptophan degradation by lactobacilli could begin in the proximal gastrointestinal tract in MCOA fed pigs and result in the observed increase in acetylated tryptophan in the small intestine of MCOA fed pigs.

The tendency of MCOA-fed pigs at D7 to have a colonic microbiota that was different than that of CON pigs, and similar to that of D14 samples, demonstrates that MCOA supports a more expedient assembly of the post-weaning colonic microbiota, resulting in a more uniform microbiota composition with fewer incidental taxa observed. This timely reassembly of the microbiota in both the small intestine and colon at D7 post-weaning is a potential benefit of MCOA, which will allow pigs to move through the vulnerable weaning period. The weaning transition period is a critical time where gut microbiota disruption can facilitate opportunistic pathogen growth resulting in post-weaning diarrhea [4,55]. There is an opportunity to improve animal health and growth outcomes during this sensitive window by assisting the pig with efficient assembly of a microbiome that is adapted to the post-weaning diet, thereby supporting pathogen exclusion, and promoting optimal immune function [1,65,66]. Improved understanding of the mechanisms by which MCOA modulate the gut microbiota can contribute to the successful application of this additive after weaning.

This paper describes the effects of a blend of medium chain fatty acids, organic acids, slow release C12, target release butyrate and a phenolic compound on microbes, metabolites, and their interactions. While the effect of blends of medium-chain fatty acids and organic acids on lactobacilli populations have been previously described [11,12,61,62,67], the effect of MCOA on bile acid dynamics offers novel insights into the mechanism by which these additives might confer benefits during the post-weaning period. The correlation between cholic acid and *Lactobacillus* relative abundance provides a possible mechanism underlying this consistently observed effect. Lastly, the relationship between *Lactobacillus* in the small intestine and tryptophan metabolism presents a new understanding of the generation of indole species in the porcine gastrointestinal tract. While these metabolites are abundant in the metabolome of pigs, it is not known where microbial tryptophan metabolism begins. Human studies of protein fermentation indicate that the colon is the primary location of microbial protein fermentation [68], however, the presence of a gastric microbiota changes the dynamics of digestion and absorption in the pig relative to these studies [69]. Finally, the relationship between MCOA, bile acid production and tissue catabolism provides an interesting area for further exploration in the weaning transition. Bile acid secretion is typically reduced post-weaning, and the effect of MCOA could allow the post-weaning pig to maintain its ability to digest and absorb lipids and, thus, protect against post-weaning catabolism. This is an interesting avenue for further research into the relationship between MCOA, and whole-body metabolism in the weaned pig.

4.5 References

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Chapter 5: High protein diets support key metabolic and intestinal functions in weaned pigs

5.1 Introduction

Despite extensive research on dietary interventions, post-weaning diarrhea remains a serious challenge to the swine industry worldwide, with significant impacts on antimicrobial use and production economics [1]. Of the most common dietary strategies currently employed is feeding diets with low crude protein (CP). Low-protein diets have been shown to decrease the risk of post-weaning diarrhea due to enterotoxigenic *E. coli* (ETEC) [2,3]. However, the abrupt transition from a high-protein (26% CP on a dry-matter basis), milk-based diet to a low-protein (13-14% CP) plant-based diet is known to detrimentally impact piglet growth performance and intestinal tissue morphology [4,5].

The role of high CP diets in post-weaning diarrhea is thought to be largely due to microbial production of protein fermentation metabolites including biogenic amines and ammonia (Blachier et al., 2021). This is supported by the diet-dependant effects of high CP, particularly relative to the availability of fermentable carbohydrate in the diet [7–9]. Dietary fibre is thought to provide beneficial effects through reduction in protein fermentation [10]. Further, it is increasingly recognized that the role of protein fermentation metabolites is complex and high concentrations of these metabolites are not always correlated with increased inflammation or disease risk [11,12].

This suggests that there is an optimal ratio of protein and fibre in post-weaning diets, which would support optimal growth and intestinal tissue function while mitigating the risk of post-weaning diarrhea in high-protein diets [10]. However, more information is needed on the integrated host-microbial mechanisms underlying these mitigating effects, and the impacts these may have on host gene expression and metabolism.

To study the effects of dietary protein and fermentable carbohydrate ratios on diet-microbe-host interactions, this study employed the use of highly controlled, semi-purified diets to study the effects of alterations in dietary protein and fibre ratios on intestinal microbiota and metabolome alongside host metabolome and transcriptome. Added fermentable carbohydrate was provided in the form of purified pectin which is highly fermentable and utilized in the distal gastrointestinal tract [13,14], increasing its effectiveness in reducing protein fermentation in the intestine. Dietary protein was increased by addition of purified potato protein. The intent of this study was to gain insight into the role of dietary protein and fibre ratios in post-weaning diets while controlling for alterations in other microbiota-shaping dietary factors such as plant polyphenols or anti-nutritional factors. Through this approach we aimed to gain further insight into alterations in physiological and metabolic response to the effect that protein and fibre ratios exert on the gut microbiota.

5.2 Materials and Methods

The animal work conducted in this study was approved by the University of Alberta Animal Care and Use Committee for livestock (AUP00003145) and conducted at the Swine Research and Technology Centre at the University of Alberta (Edmonton, Canada).

Diets

To determine the effects of protein and fermentable fibre on diarrhea, microbiota composition and metabolome four semi-purified diets were used. The dietary treatments were control (CON), high-protein (HP), high-fibre (HF), and high-protein with high-fibre (HFHP). The control diet was formulated to meet current industry recommendations for protein level post-weaning. Diets were formulated to be isoenergetic with comparable standard ileal digestibility of lysine, methionine, threonine, and tryptophan. Diets all met or exceeded nutrient requirements for

weaned pigs. Diets were formulated using a wheat and soybean meal base (Table 5.1), where dietary fibre was increased through inclusion of highly fermentable purified pectin (Pectin 64017, Cargill, USA), while dietary protein was increased using purified potato protein (Protastar, Avebe, Veendam, the Netherlands).

Table 5.1. Composition of experimental diets.

Ingredients, %	CON	HF	HP	HFHP
Wheat	40	40	40	40
Whey Permeate/ 80% lactose	6	6	6	6
Soybean Meal (dehulled solvent)	12	12	12	12
Corn Starch	16.34	17.42	11.55	9.21
HP300	2	2	2	2
Herring meal	6	6	0	0
Canola oil	4	4	4	4
Potato Protein (Protastar)	0	0	14	14
Pectin	0	7	0	7
Limestone / glass rock	0.9	0.9	1.22	1.22
Mono/dical phoshate	1	1	1.8	1.8
Salt	1	1	1	1
L-Lysine HCl	0.91	0.93	0.25	0.2
L-Threonine	0.4	0.4	0	0
DL-Methionine	0.18	0.18	0.03	0.03
L-Tryptophan	0.08	0.08	0	0
AAF pig vit px dextrose 5kg/tonne	0.5	0.05	0.5	0.5
AAF pig tm px dextrose 5kg/tonne	0.5	0.5	0.5	0.5
Chromic Oxide	0.5	0.5	0.5	0.5
Choline Chloride 60%	0.03	0.03	0.03	0.03
Celite	7.65	-	4.61	-
Phytase	0.01	0.01	0.01	0.01

Animal Selection and Husbandry

At weaning, pigs were weighed and 40 male piglets representing the average weight for their litter were selected. Prior to the start of experimental diets, 40 male pigs were transferred to metabolic pens for a 7-day acclimation period where they were housed with a litter-mate companion. During this time pigs were fed a standard post-weaning diet. Throughout acclimation and the experimental period, photoperiod was maintained on a 12-hour artificial lighting schedule. Room temperature, humidity, and ventilation were controlled using a proportional environmental controller. Feed intake, measured as feed disappearance, was measured twice daily. On experimental day zero (D0), pigs were weighed and assigned to one of four dietary treatments (Table 5.1). Treatment groups were balanced for body weight and litter of origin. Following selection, companion piglets were removed, and diets were abruptly changed to mimic weaning stressors.

To standardize time of feed intake prior to euthanasia, feed was restricted overnight on day 6 to 0.75 of average overnight feed intake. On the morning of experimental day 7 feed was removed as the light period began and pigs were refed 3 hours prior to scheduled euthanasia.

Sample Collection

Body weights were collected at experimental D0 and D7 to measure growth and for calculations of feed efficiency. Fresh fecal samples were collected on day 3 for use in fecal dry matter assays as measure of diarrhea. On experimental day 7, blood samples were collected for metabolomics analysis. Following this, pigs were euthanized and tissue and digesta collection was performed. Briefly, individual segments of the digestive tract were clamped to prevent mixing and samples were collected from the terminal ileum (10 cm proximal to ileocecal junction) and the cecum. Contents of the cecum were thoroughly mixed prior to pH measurement and digesta

collection. Digesta samples were snap frozen in liquid nitrogen and stored at -80 °C until further use. Tissue samples from the cecal tip were dissected with removal of any lymph nodes and digesta before snap freezing for subsequent transcriptomic analysis.

Diarrhea Prevalence

As part of routine health checks, diarrhea was monitored for each pig twice daily using a fecal scoring system from 1 (firm and dry pellets) to 8 (watery liquid with slight brown or yellow tinge). Pigs were considered to have diarrhea if their fecal score was greater than or equal to 6 (feces has no defined shape, present in piles and leaves residue when picked up). To analyze diarrhea prevalence, fecal scores for each pig on Day 3, 5 and 7 were compared. A fecal sample was also collected on day 3 and fecal water content was determined using a loss on drying protocol. A 1-gram sample of fresh feces from each pig was weighed and placed in a forced air oven at 135 °C for 2 hours. Following drying, moisture content was calculated as the weight lost on drying divided by the test sample weight. All treatments were compared using ANOVA with a Tukey HSD comparison of means in R.

Microbiota Composition Analysis

Total DNA was extracted from digesta using the QIAmp DNA Stool Mini kit (Qiagen, Montreal, QC) with the addition of a bead beating step [15]. A FastPrep-24^{TM} homogenizer (MP Biomedicals, OH, USA) was used and bead beating was performed for 45 s at 6m/s. DNA concentration was measured using the Quant-iT PicoGreen dsDNA kit (Invitrogen, CA,USA) following extraction. The V3/V4 region of 16S rRNA was sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) following the Illumina protocol for amplicon library construction (16S Metagenomic Sequencing Library Preparation), and using the following primers:

Forward:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’

Reverse:

5'-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’

Sequences were processed through a quality control pipeline including denoising, demultiplexing, and trimming sequences with low quality scores in QIIME2 [16] and a phylogenetic tree generated. Samples were rarefied to an even read depth for alpha diversity analysis. Alpha diversity was measured using Faith’s Phylogenetic Diversity and Shannon diversity index. Samples with low read-depth were removed from the dataset for beta diversity analysis but data were not rarefied. Beta diversity was measured using weighted and unweighted UniFrac measures and differences were determined by PERMANOVA with the addition of pairwise comparisons. β-diversity visualization was performed in R using the phyloseq package [17]. Amplicon sequence variant tables (ASVs) for both locations were imported into R and differentially abundant ASVs between treatment groups were identified using DESeq2 and visualized using the ComplexHeatmap package in R [18,19]. Genera-level differences were examined by collapsing ASVs using phyloseq and evaluated using DESeq2.

Metabolomics

Metabolites in serum, ileal digesta and cecal digesta were quantified using the TMIC Prime Assay (The Metabolomics Innovation Centre, Edmonton, Alberta). Data analysis was performed using Metaboanalyst 5.0 [20]. Data were processed using median normalization and range scaling. Global metabolite changes for each compartment were examined using ANOVA and a Fisher’s LSD comparison of means with an FDR correction for multiple comparisons. Significantly different metabolites identified during metabolomics analysis for each compartment were further

evaluated for metabolic pathway differences using the quantitative enrichment analysis function in MetaboAnalyst.

Transcriptomic Analysis

To examine the effects of different combinations of protein and fibre on intestinal tissue gene expression, RNA-Seq analysis was performed on cecal tissue. Samples from the cecal tip of each animal were ground individually under liquid nitrogen and submitted for RNA extraction, library construction, and mRNA sequencing at the Genome Quebec Centre D'expertise et De Services (Montreal, Quebec, Canada). Library construction was performed using the Illumina protocol for library construction and sequencing performed using the Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA). Raw sequencing reads were processed using the nfcore/rnaseq (3.6) pipeline for stranded reads [21]. Reads were mapped to the *Sus scrofa* reference genome 10.2 [22]. The reference genome and annotation files were obtained from Ensembl release 81 [23], mapped using STAR (2.6.1d) [24] and quantified using Salmon (1.5.2) [25]. Length-scaled counts of annotated transcripts were interrogated for differentially expressed genes were identified in R using edgeR (3.38.4) [26], significant differences between treatment groups were identified using FDR-adjusted p-values.

5.3 Results

Performance

Pigs on the HP diet showed higher average daily gain (ADG) over the course of the 7-day experiment compared to both CON and HF groups. An intermediate growth phenotype was observed in HFHP animals. However, when feed efficiency (measured as feed to gain or F:G) was compared, better performance was observed in both HP and HFHP pigs compared to pigs fed CON or HF diets (Figure 5.1).

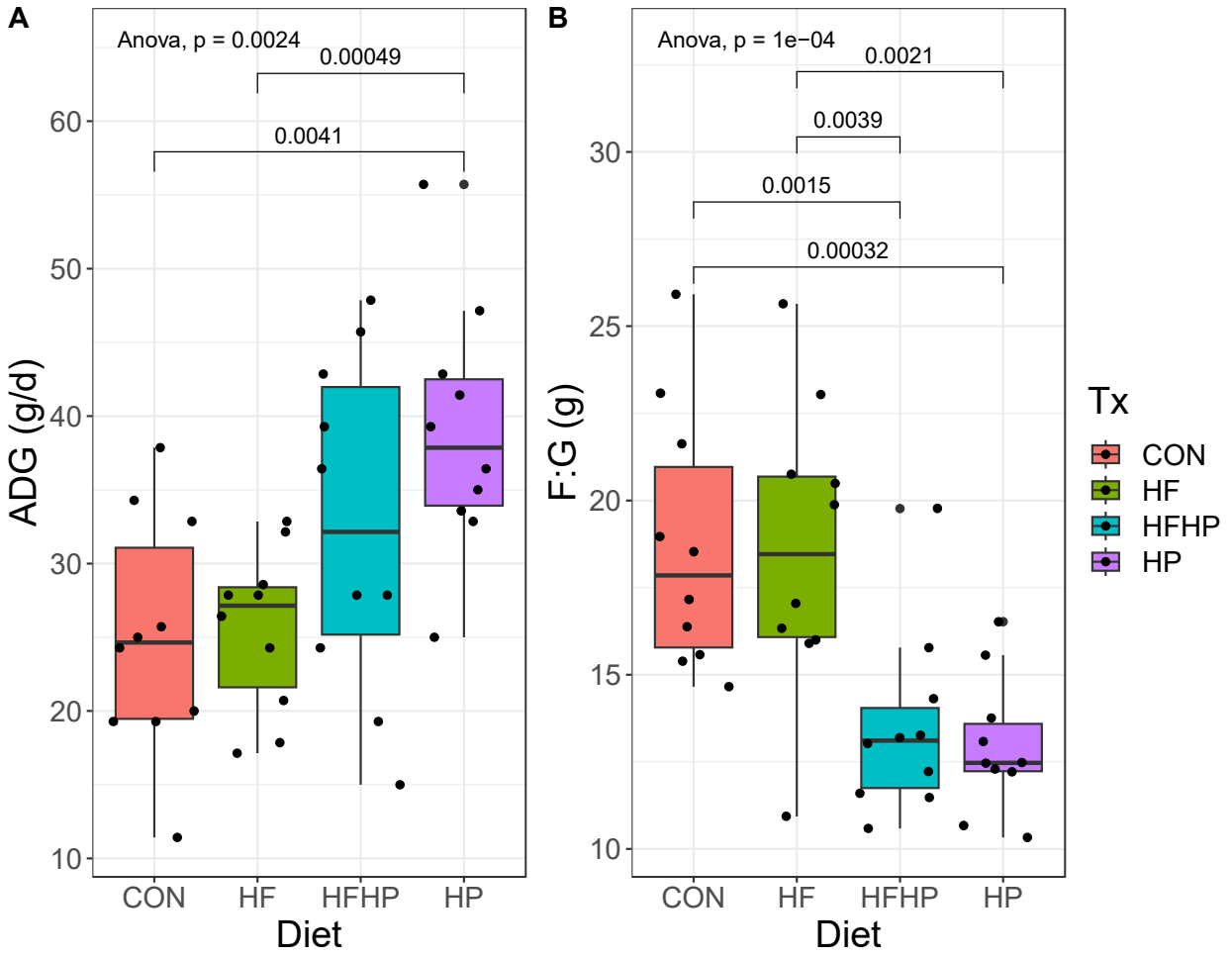

Figure 5.1. Growth performance and feed efficiency data for pigs fed a standard post-weaning diet (CON) or a diet with added fibre (HF), protein (HP) or both (HFHP) for 7 days. No significant differences in average daily feed intake (ADFI) were observed between the diets. Significant differences were detected using ANOVA with a Tukey's HSD comparison of means, p-values are shown for all significantly different comparisons.

When diarrhea prevalence was compared using fecal scores (Figure 5.2), no differences in diarrhea prevalence were detected between treatment groups (P = 0.32-0.63). Fecal dry matter analysis on D3 showed a decrease in fecal dry matter between HF and HFHP compared to CON animals (Figure 5.3). This difference was not observed for HP diets. Consistent with the gel

forming characteristics of pectin, the high-fibre diets resulted in an increase in fecal water, whereas inclusion of high-protein had no impact on mean fecal water content.

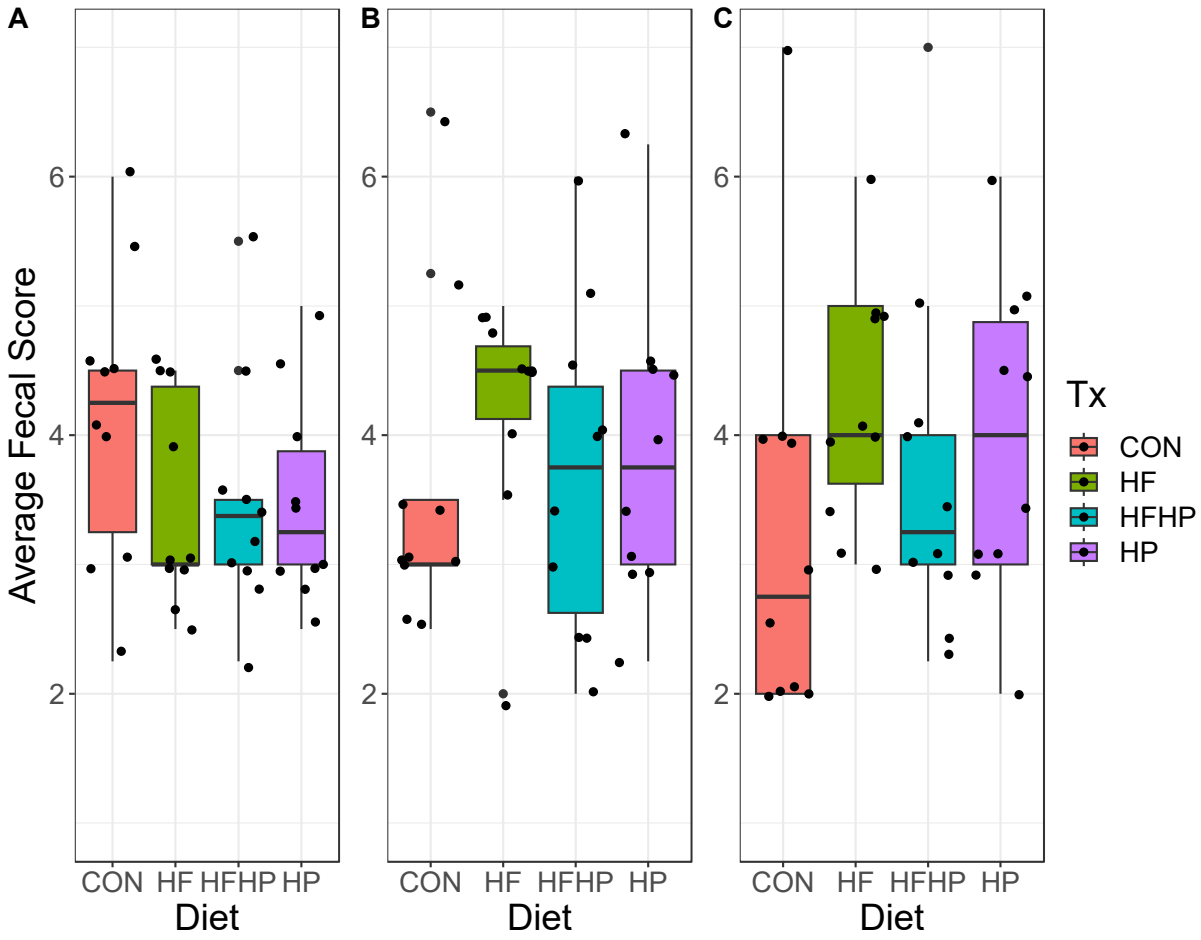

Figure 5.2. Average Daily Fecal score of pigs fed a standard post-weaning diet (CON) or a diet with added fibre (HF), protein (HP) or both (HFHP) on **A.** experimental D3; **B.** D5; **C.** D7. No significant differences in fecal score were detected during the duration of the experiment. Fecal scores of 6 or greater are indicative of diarrhea.

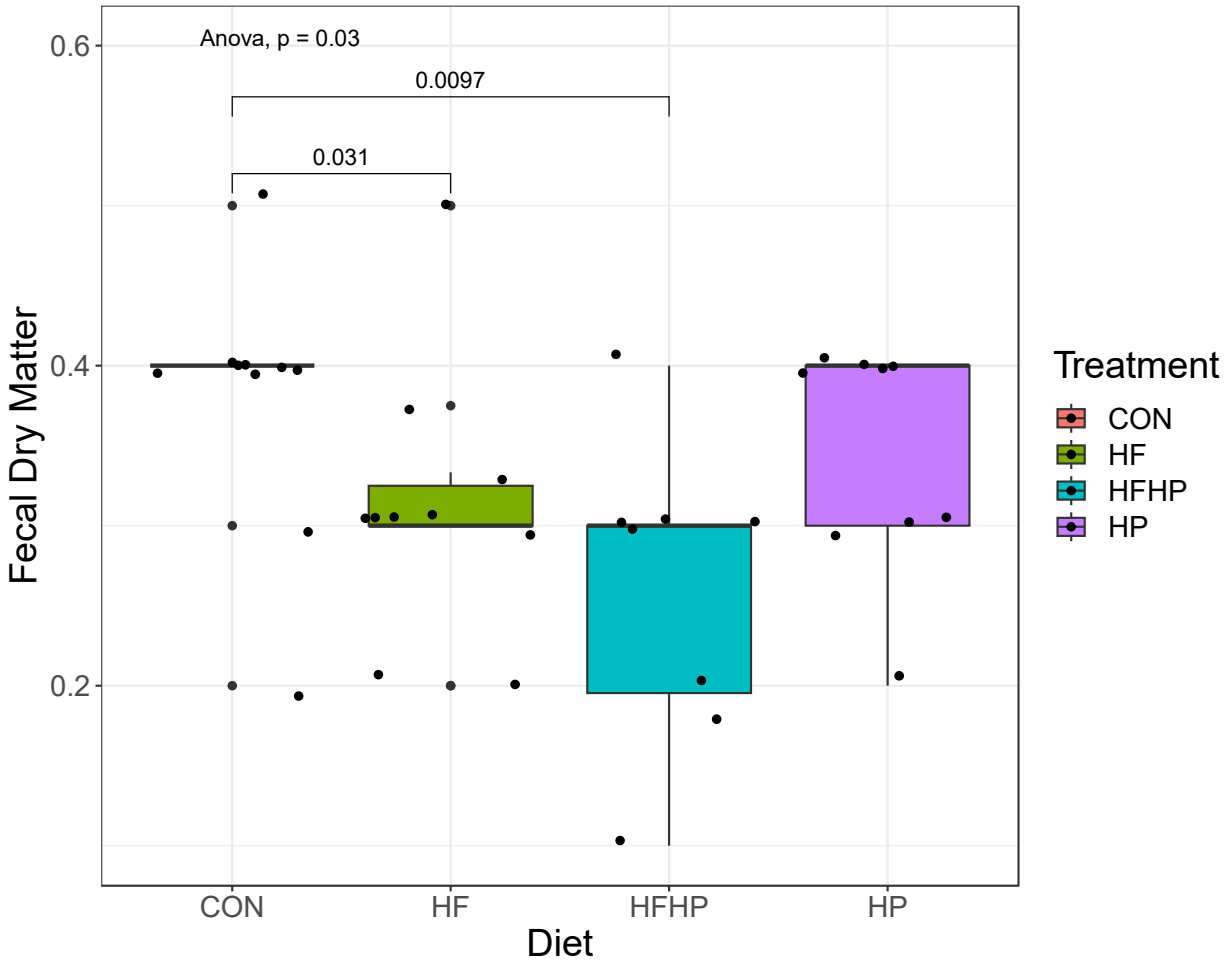

Figure 5.3. Fecal dry-matter content of pigs fed a standard post-weaning diet (CON) or a diet with added fibre (HF), protein (HP) or both (HFHP) on experimental D3. Significant differences in dry matter were detected using ANOVA with a Tukey's HSD comparison of means, p-values are shown for all significantly different comparisons.

Microbiota

Ileal microbiota composition did not differ for either measure of β-diversity used (Figure 5.4a). Shannon indices of α-diversity also showed no differences in community richness and evenness in the ileal microbiota between the different treatments (Figure 5.4b).

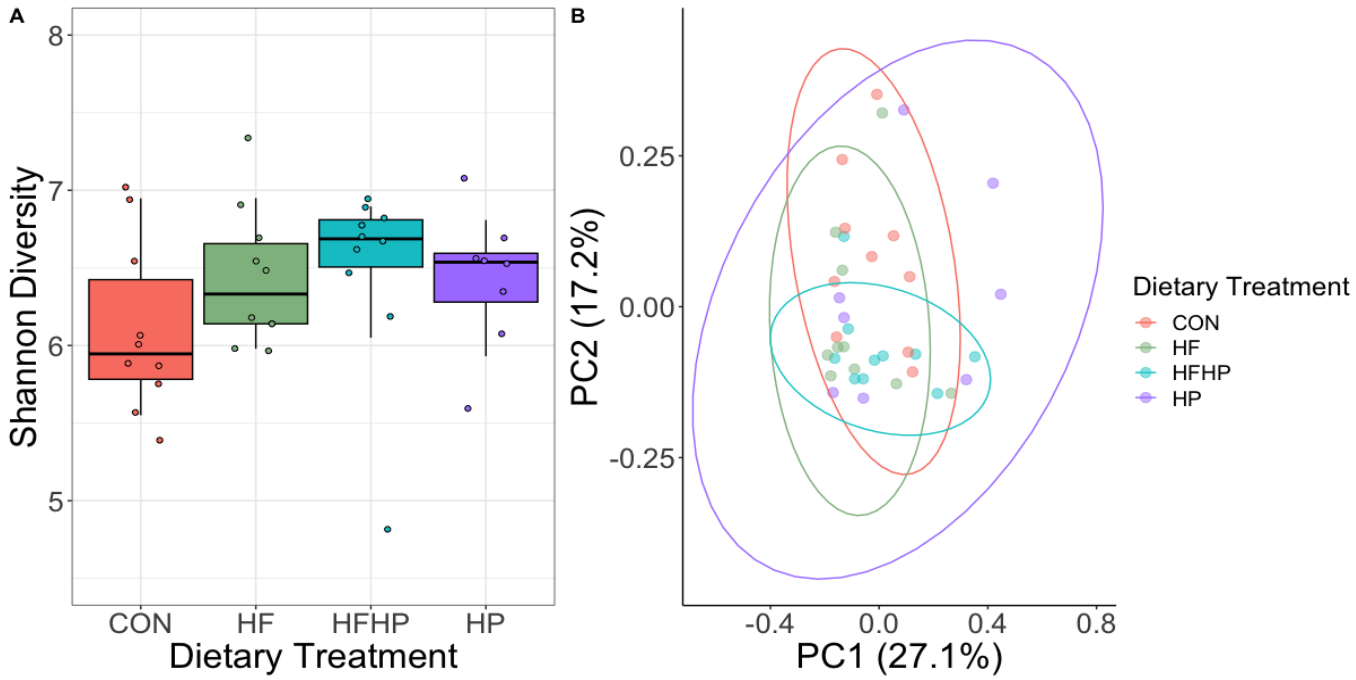

Figure 5.4. Measures of ileal microbiota structure after 7 days of a standard post-weaning diet (CON) or a diet containing added fibre (HF), added protein (HP), or both (HFHP). No significant differences were detected in α- or β-diversity including measures of **A.** unweighted UniFrac distance or **B.** Shannon diversity.

Comparison of cecal microbiota composition showed differences in both α- and β-diversity metrics. Both weighted and unweighted UniFrac measures of β-diversity, indicated differences between groups ($P = 0.008$ and $P = 0.01$ respectively, Figure 5.5). Dispersion measured using the permdisp function in QIIME2 was not different between any of the groups for either metric (P = 0.11-0.95). Pairwise comparison further revealed that community composition of the cecal microbiota CON-fed pigs was different than all others when evaluated using weighted UniFrac, and different from the HFHP diet when unweighted UniFrac was considered (Table 5.2). The HP diet differed from the HF diet on both measures of β-diversity (Table 5.2) and tended to differ from the HFHP diet for unweighted UniFrac only (P = 0.05). Alpha-diversity, measured using Shannon diversity index, showed a decrease in alpha diversity for both HF and HFHP compared to the CON or HP diets (Figure 5.5c).

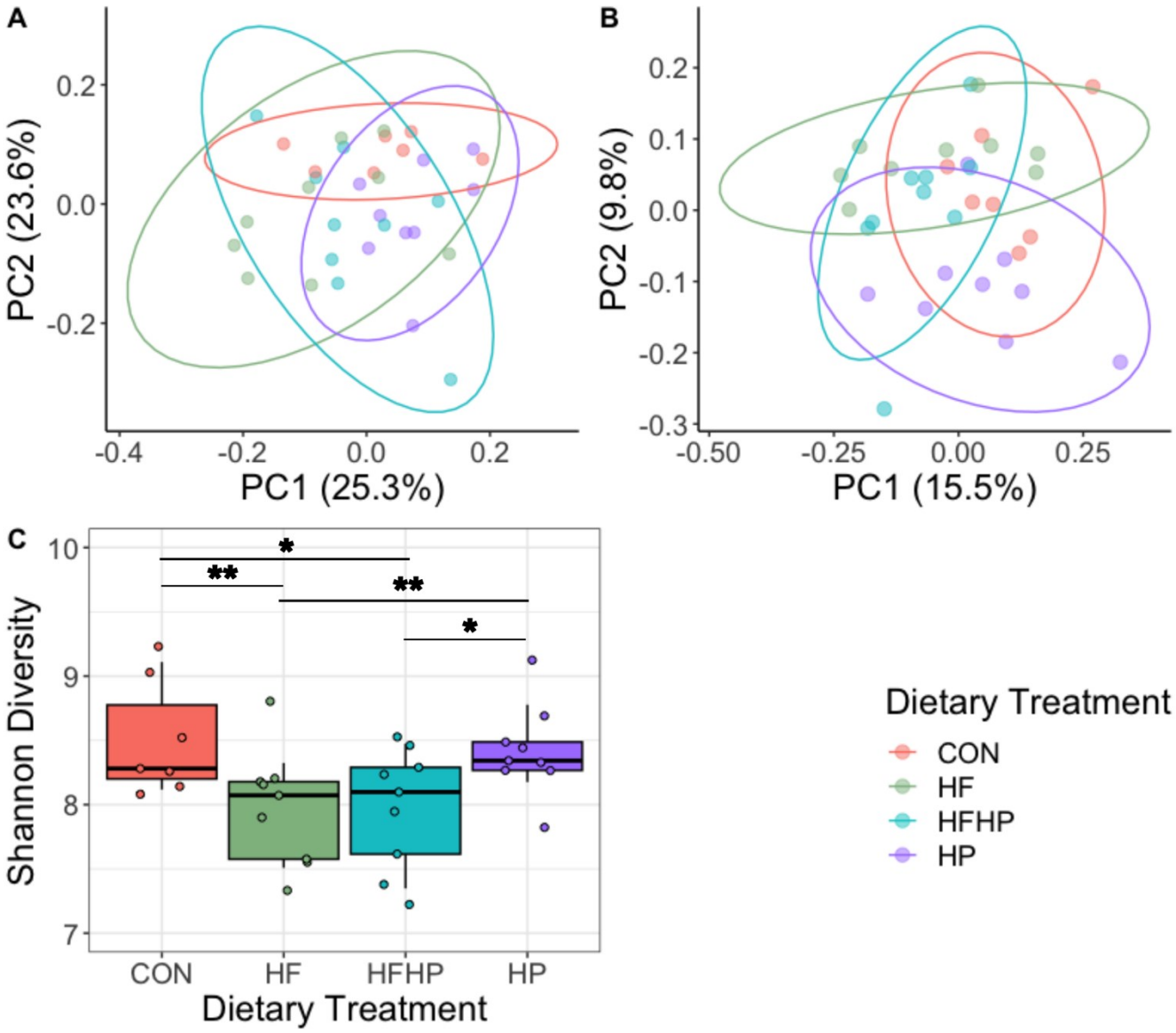

Figure 5.5. Differences in cecal microbiota α- and β-diversity after 7 days of a standard post-weaning diet (CON) or a diet containing added fibre (HF), added protein (HP), or both (HFHP). A significant difference in a. weighted UniFrac distance (P = 0.008) and b. unweighted UniFrac distance (P = 0.01) were both detected for unrarefied microbiota data. c. Significant differences (P < 0.05) and statistical trends (P < 0.10) in Shannon index of α-diversity were also observed and are denoted with ** and * respectively.

Table 5.2. Pairwise comparison of cecal microbiota *β*-diversity.

Comparison	P-value	
	Unweighted UniFrac	Weighted UniFrac
CON-HF	0.15	0.02
CON-HFHP	0.02	0.02
CON-HP	0.21	0.01
HF-HFHP	0.93	0.38
HF-HP	0.005	0.004
HFHP-HP	0.05	0.2

Amplicon sequence variant-level differences were compared using DESeq2 in R, identifying 198 differentially abundant ASVs between diets. The top-20 differentially abundant ASVs were visualized by heatmap, primarily showing a difference between the CON diet compared to all others. Of interest within this subset of highly different ASVs was the increased presence of ASVs belonging to the genera *Prevotella* and *Clostridium* (Figure 5.6). However, when the ASVs were collapsed into identified genera, these individual ASV differences did not result in an overall shift in the abundance of *Prevotella* or *Clostridium*. Of the 54 genera identified within the samples, significant differences in 4 genera were identified (Figure 5.7). These differences were in part due to two genera with high abundance in the cecal microbiota of a small number of individuals within a group (*Weisella* and Peptococcaceae rc4-4). Two other differentially abundant taxa, *Bulleida* p-1630-c5 and *Butyricicoccus pullicaecorum*, were detected with higher frequency. *Butyricicoccus pullicaecorum* was significantly lower in the HP-fed pigs compared to all other groups based on DESeq contrasts of count data. However, it is of note that aside from the HF-fed group where the median relative abundance of this species is 23%, the relative abundance in all other groups was lower than 2%. *Bulleida* p-1630-c5 abundance was affected by dietary fibre with both the CON and HP diets differing from the HF and HFHP diets. This taxon is also lowly abundant, with the highest relative abundance detected in the HF and HFHP diets at 1.2% of the cecal microbiota.

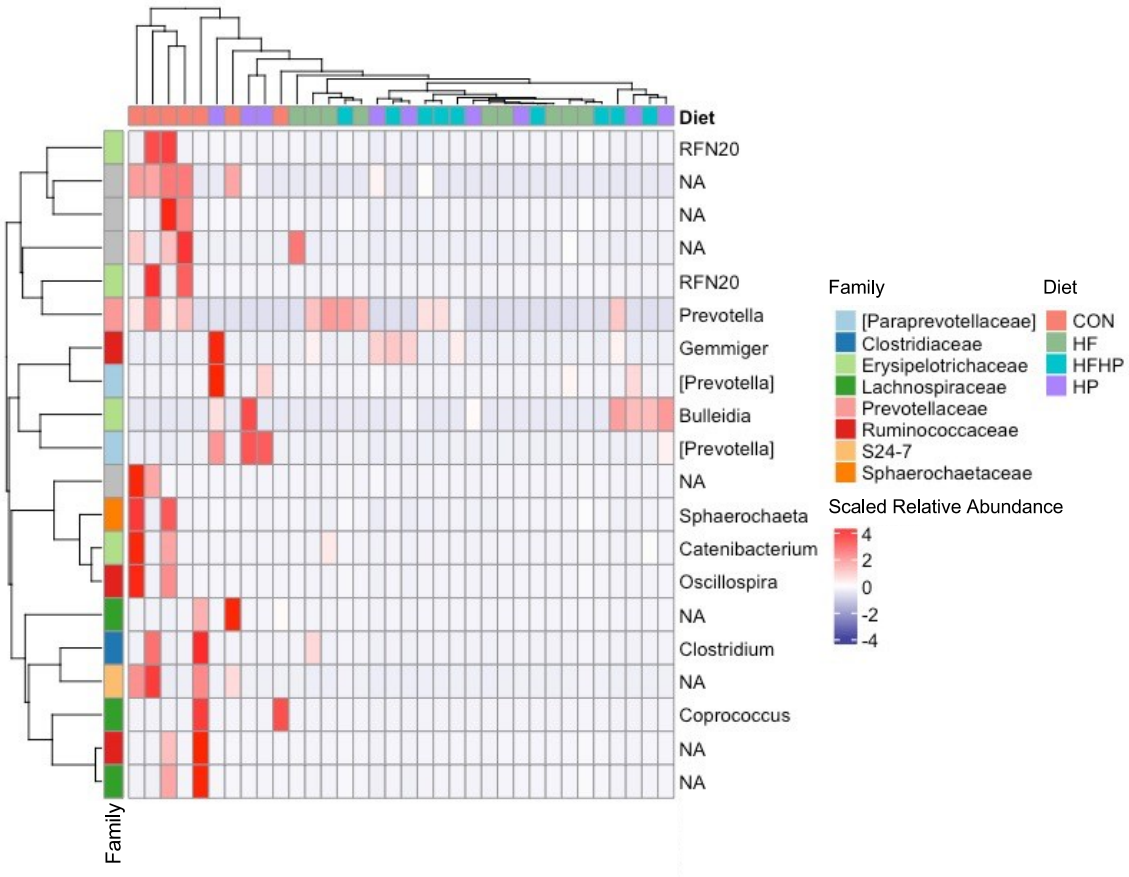

Figure 5.6. Heatmap of differentially abundant ASVs identified in the cecal microbiota of pigs fed a standard post-weaning diet (CON) or a diet containing added fibre (HF), added protein (HP), or both (HFHP) for 7 days. Differences were identified at the ASV level using DESeq2 in R. Colour scale represents the within row (ASV) scaled relative abundance. Rows with the label NA represent ASVs which were not classified below the family level.

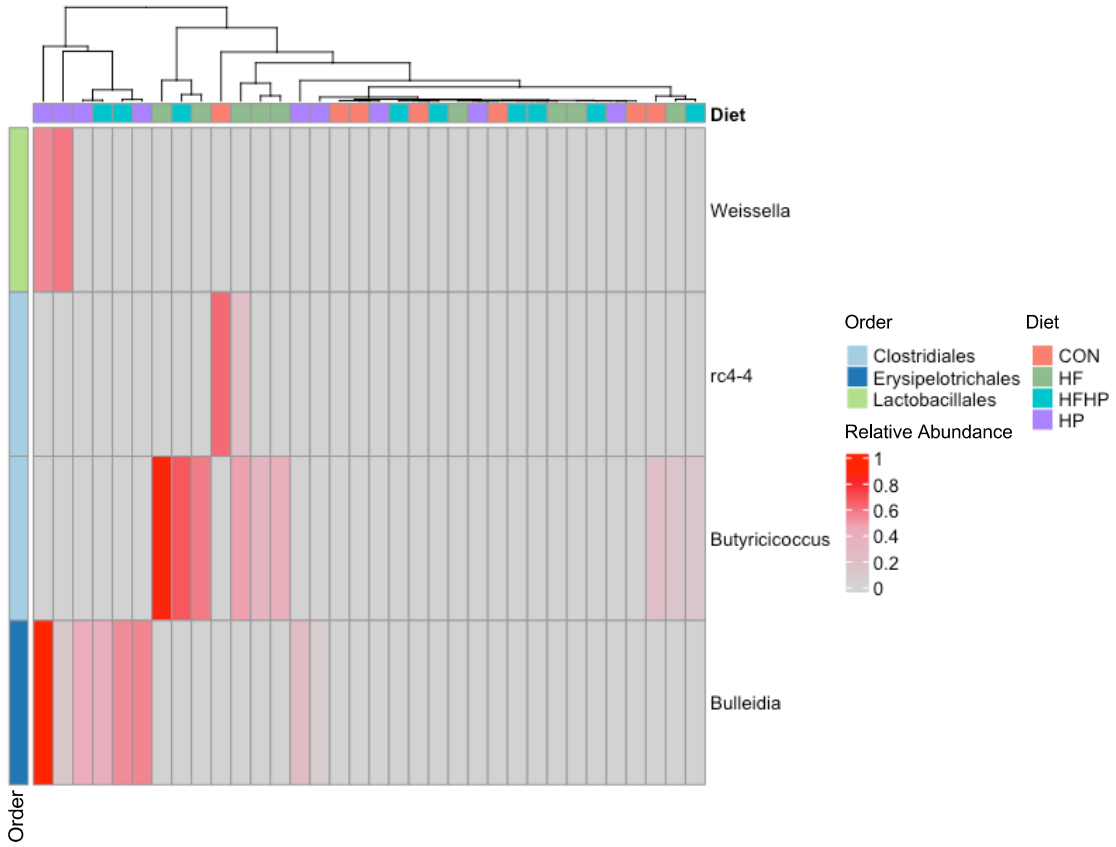

Figure 5.7. Heatmap of differentially abundant microbial genera identified in the cecal microbiota of pigs fed a standard post-weaning diet (CON) or a diet containing added fibre (HF), added protein (HP), or both (HFHP) for 7 days. ASVs were collapsed into known genera using phyloseq, and differential abundance examined using DESeq2 in R. Colour scale represents the within row (Genus) relative abundance and was not scaled.

Ileal Metabolome

While there was substantial overlap in the ileal metabolome (Figure 5.8a) of the four groups for the compounds evaluated upon examination with principal component analysis (PCA), 13 compounds were identified as significantly different between groups using ANOVA with Fisher's LSD comparison of means (Figure 5.8b). The pattern of separation in these metabolites showed a predominant effect of dietary protein. Increased methionine-sulfoxide and acylcarnitines were observed in both high-protein diets. The increased dietary fibre in the HFHP diet also resulted in

some uniquely enriched metabolites, betaine, glutamine, and glutamic acid compared to all other diets. Low protein diets (CON and HF) displayed increased taurine, trans-hydroxyproline, and a trend toward increased creatinine (P = 0.07, Figure 5.9) alongside increased cell-membrane components in the form of sphingomyelin and glycerophospholipids.

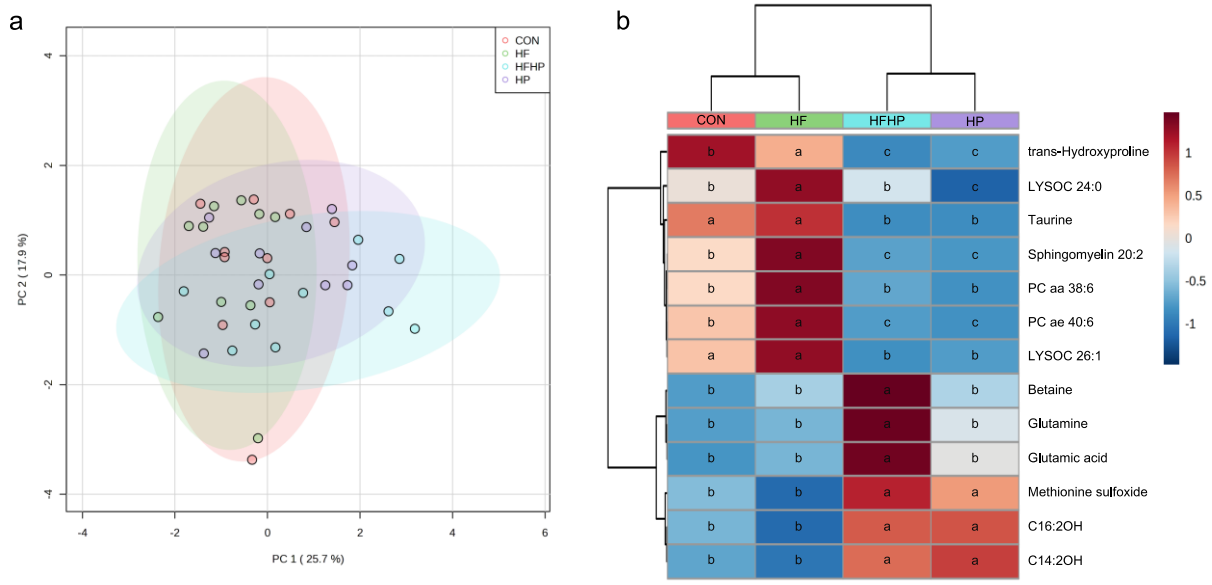

Figure 5.8. Metabolomic differences in ileal digesta of animals fed a standard post weaning diet (CON), or a diet with high-fibre (HF), high-fibre and high-protein (HFHP) or high-protein (HP) diet for seven days. a. PCA of all metabolites examined and b. heatmap of metabolites found to be significantly different by ANOVA. Within-row letters indicate significant differences between means identified by Fisher's LSD. Metabolomics analysis was performed using the quantitative TMIC prime assay and statistical analysis performed in Metaboanalyst 5.0. Colour scale was generated using the Z-scores for each row.

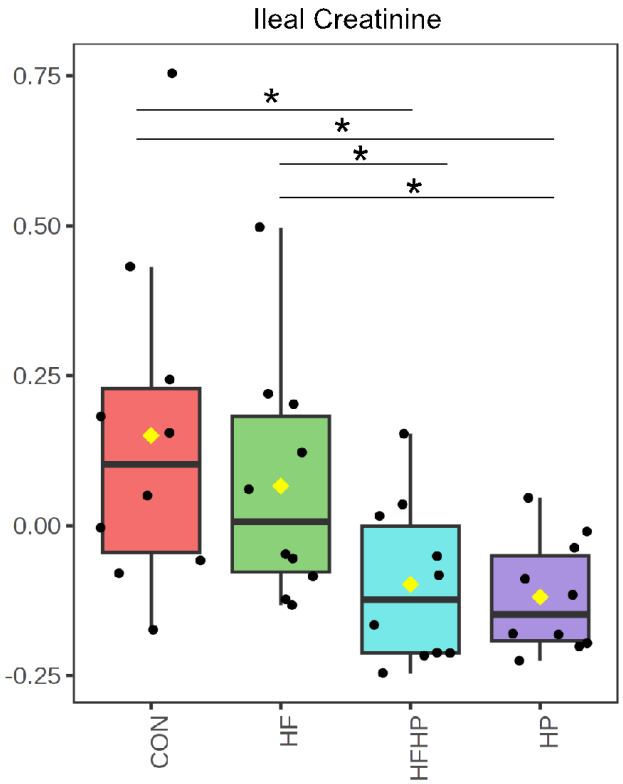

Figure 5.9 Shannon index of α-diversity. Normalized concentrations of creatinine detected in the ileal digesta of pigs fed a standard post weaning diet (CON), or a diet with high-fibre (HF), high-fibre and high-protein (HFHP) or high-protein (HP) diet for seven days. Y-axis values represent scaled concentrations compared to the overall mean plasma creatinine observed. Statistical trends ($P < 0.1$) are noted, no statistically significant differences were detected ($P < 0.05$).

Cecal Metabolome

Similar to the ileal metabolome, substantial overlap in the cecal metabolome was observed between diets (Figure 5.10a). Analysis of cecal metabolites identified 10 significantly different metabolites after FDR-adjustment (Figure 5.10b). These metabolites largely separate based on dietary protein level where increased acylcarnitine species are observed in the cecum for pigs fed diets containing higher protein levels. The lower-protein diets showed higher levels of sphingomyelin and glycerophospholipids. These shared patterns are suggestive of a conserved effect of dietary protein on intestinal metabolome between the two locations.

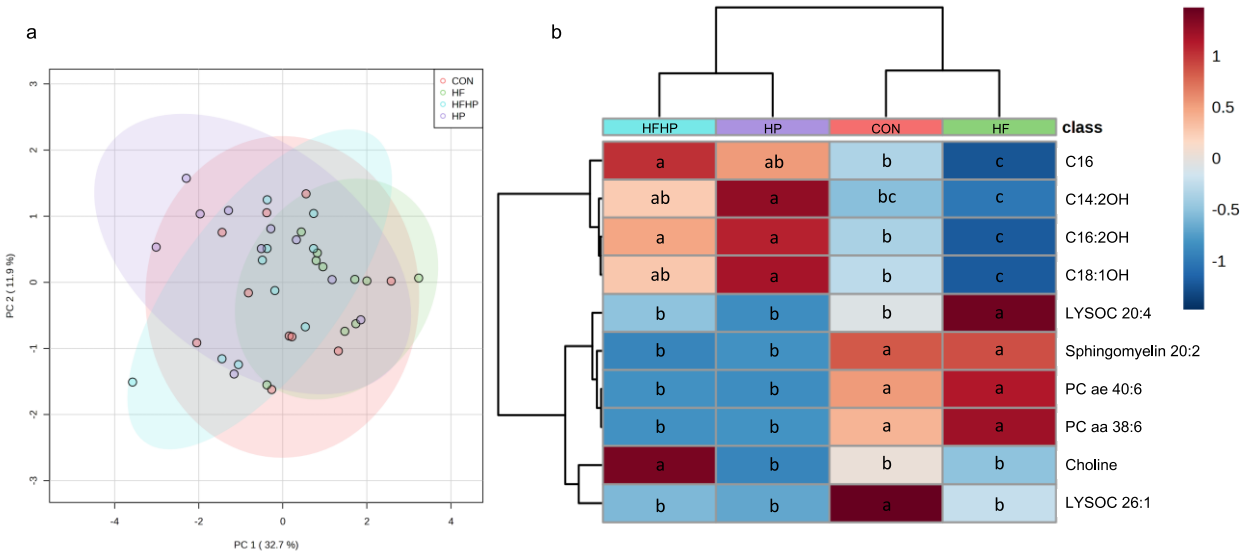

Figure 5.10. Metabolomic differences in cecal digesta of animals fed a standard post weaning diet (CON), or a diet with high-fibre (HF), high-fibre and high-protein (HFHP) or high-protein (HP) diet for seven days. a. PCA of all metabolites examined and b. heatmap of metabolites found to be significantly different by ANOVA. Within-row letters indicate significant differences between means identified by Fisher's LSD. Colour scale was generated using the Z-scores for each row.

Serum Metabolome

Marked differences between high- and low-protein diets were observed in the overall serum metabolome when visualized using PCA (Figure 5.11a). A total of 68 metabolites were found to be different between groups including many amino acids and glycerophospholipids, the top 25 of which were also visualized by heatmap (Figure 5.11b). An increase in spermidine in the HP diet is indicative of protein fermentation by microbes, while this effect was mitigated with addition of dietary fibre in the HFHP diet. This effect was also observed in the HF det compared to the control. In low-protein diets, increased glycerophospholipids and metabolites indicative of shifts in amino acid metabolism such as lysine and amino-adipic acid were found to be increased.

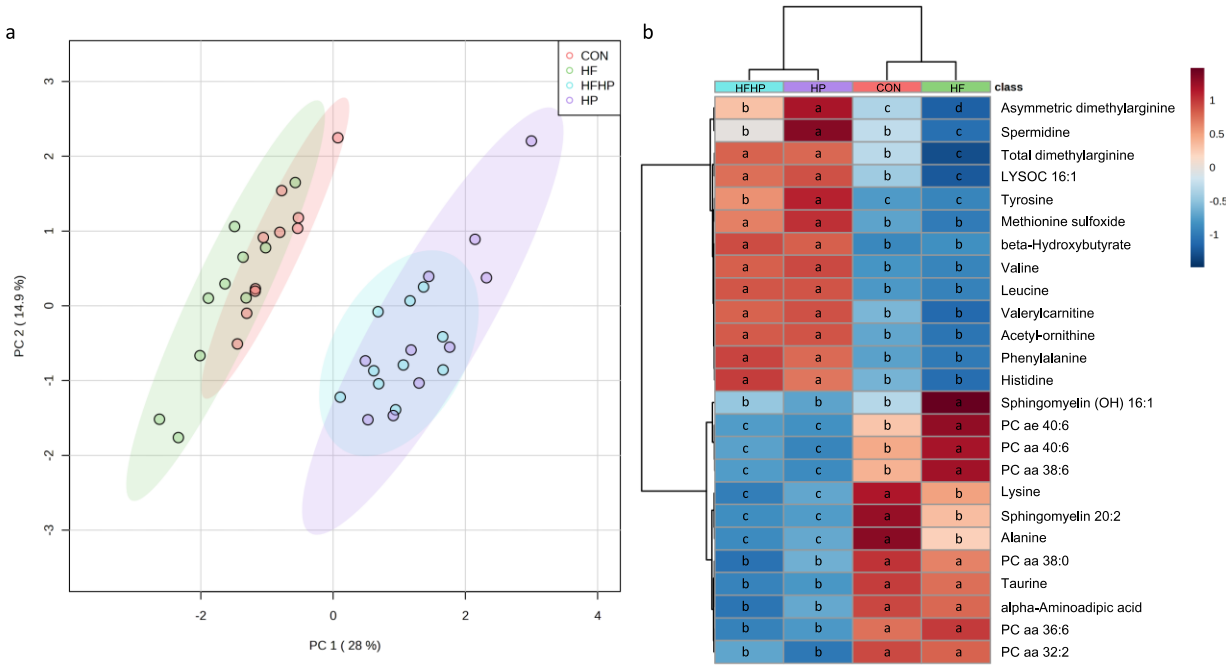

Figure 5.11. Metabolomic differences in jugular serum of animals fed a standard post weaning diet (CON), or a diet with high-fibre (HF), high-fibre and high-protein (HFHP) or high-protein (HP) diet for seven days. a.PCA of all metabolites examined and b. heatmap of metabolites found to be significantly different by ANOVA. Within-row letters indicate significant differences between means identified by Fisher's LSD. Colour scale was generated using the Z-scores for each row.

As protein was the key driver of serum metabolome differences, metabolic pathway analysis of serum metabolites for the HP vs. CON diet was performed by quantitative enrichment analysis (QEA). Twelve significantly enriched pathways were detected in HP-fed pigs including for fatty acid biosynthesis, propanoate metabolism and many amino acid biosynthesis pathways (Figure 5.12a). Five significantly enriched pathways identified in CON pigs included carnitine synthesis, phospholipid biosynthesis, lysine degradation, biotin metabolism, and tryptophan metabolism (Figure 5.12b). Within the amino acid metabolism pathways, differences in glycine, serine, arginine, and proline metabolism were detected with many metabolites within this network being different (Figure 5.13).

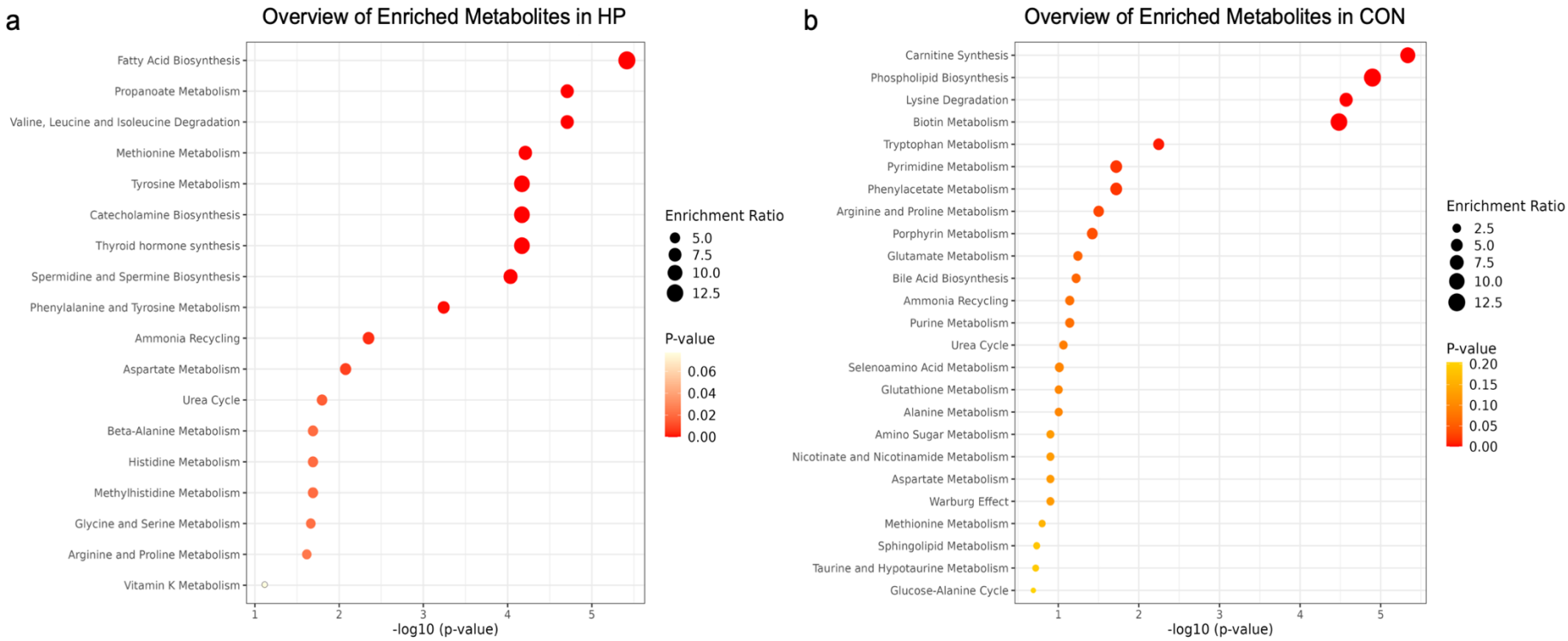

Figure 5.12. Quantitative enrichment analysis results for metabolic pathways between pigs fed **a.** high-protein and **b.** control diets.

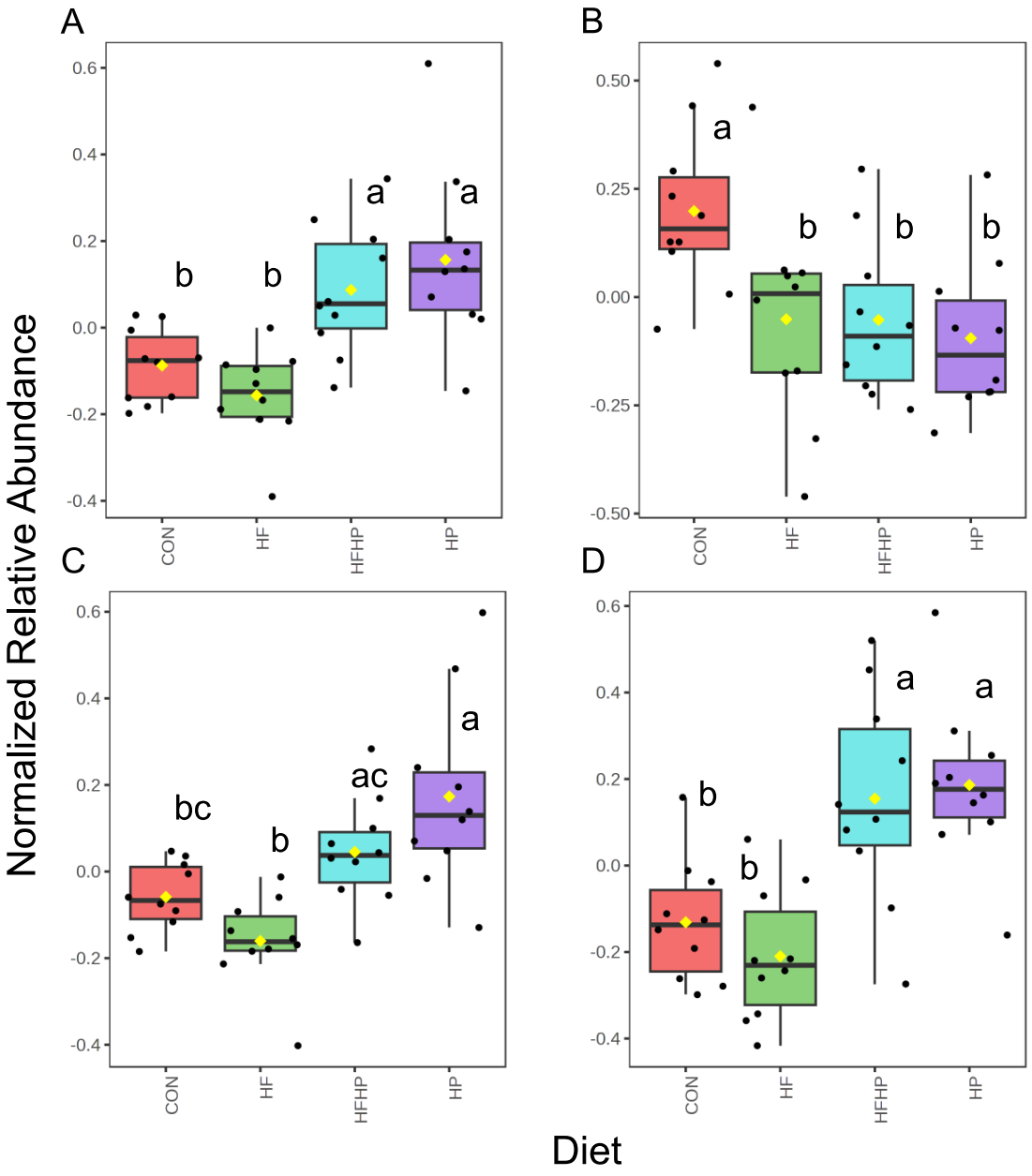

Figure 5.13. Alterations in serine, glycine and arginine metabolism. Serum metabolites associated with serine, glycine and arginine metabolism observed after 7 days for animals fed a standard post weaning diet (CON), or a diet with high-fibre (HF), high-fibre and high-protein (HFHP) or high-protein (HP) diet. **A.** Arginine; **B.** Glutamate; **C.** Ornithine **D.** Citrulline.

Transcriptomics

After FDR-correction, transcriptomics revealed only 7 differentially expressed (DE) transcripts in the cecal tissue (Figure 5.14). The most marked transcriptomic differences were between the HP-fed pigs and those fed a CON or HF diet. Of these DE genes, 6 of 7 were found to be decreased in high-protein diets (HP or HFHP) compared to low-protein diets (CON or HF). These included genes involved in serine synthesis (PSPH and PSAT1) and cellular stress response (CHAC1, ASNS, ADM2, GPT2). While one DE gene, ABHD4, with important roles in lipid metabolism and satiety was increased in high-protein diets. Only one differentially expressed transcript was identified between the CON- and HF-fed pigs (ADM2), while no transcripts differed between HP- and HFHP-fed pigs. The difference in expression of serine synthesis pathway genes followed a shared pattern of decreased expression in both high-protein diets with large fold change differences, particularly in PSAT1 which was found to have $\log_{10}$ fold change decreases of 1.90 to 3.15 in high protein-fed animals. This consistent pattern of dietary protein effects was also observed for three of the cellular stress response genes, ASNS, CHAC1, and GPT2, with $\log_{10}$ fold change decreases of 0.92 to 2.4 in pigs on high-protein diets. The expression of ADM2 followed the same pattern, however, the HF animals were intermediate between the CON and high-protein diets which may be of interest given the role of ADM2 in adipose tissue homeostasis and insulin sensitivity. Increased expression of ABHD4 observed in the high protein-fed animals was more subtle, with smaller changes observed ($\log_{10}$ fold change 0.64-084). These patterns demonstrate that at comparable levels of protein, dietary fibre was not a key driver of transcriptomic changes in the cecum, while a more pronounced effect of dietary protein on cecal gene expression was seen.

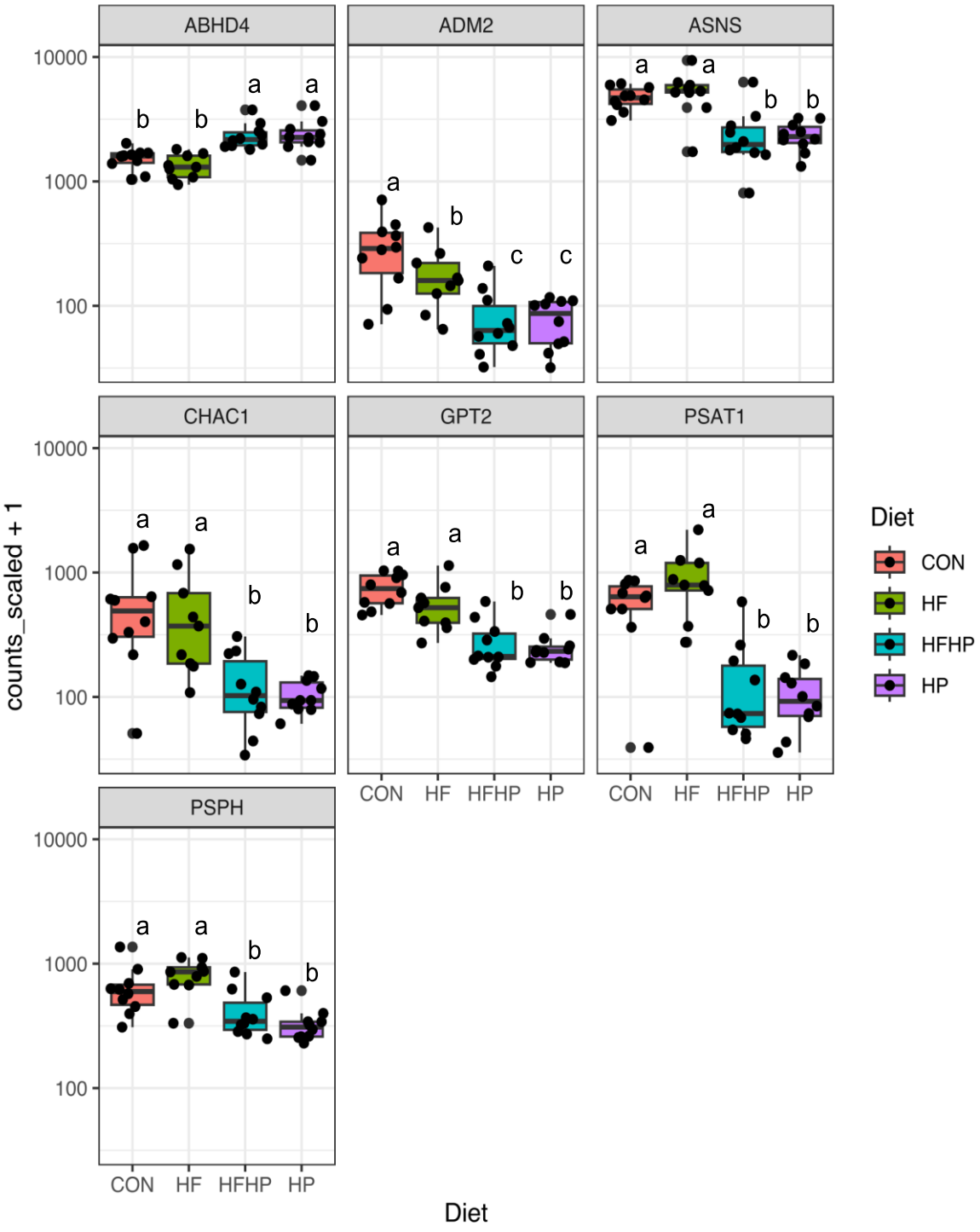

Figure 5.14. Differentially expressed transcripts identified in the cecal tissue of weaned pigs after 7 days on a standard post weaning diet (CON), or a diet with high-fibre (HF), high-fibre and high-protein (HFHP) or high-protein (HP) diet.

5.4 Discussion

Through the multi-omics techniques utilized, many changes in host response to dietary protein were identified. Results of the metabolomic and transcriptomic analyses demonstrated an important role for increased dietary protein in improving amino acid metabolism linked to muscle accretion, and intestinal tissue homeostasis post-weaning. Low-protein diets on the other hand resulted in increases in digesta phospholipids and sphingolipids suggesting increased bile secretion, and may be linked to serine deprivation responses detected in cecal tissue. Unlike previous studies, pronounced differences in the microbiota were not observed and detrimental effects of high-protein diets were not identified. Taken together, these results show increased support of key processes involved in weaned pig growth and intestinal tissue function on a higher protein diet, and suggest that on a low-protein diet, many key cellular processes are detrimentally affected.

Impacts of dietary protein level on amino acid and muscle tissue metabolism

In both the ileum and serum, differences in amino acids and their metabolites were detected. While most of the increased free amino acids (phenylalanine, histidine, leucine, valine, and tyrosine) detected in the serum of HP and HFHP pigs likely reflect dietary differences, alterations in metabolites from the urea cycle, lysine degradation, glycine, serine, and methionine metabolism were also detected.

Perhaps of most interest in the context of growth performance, pigs fed low protein diets displayed increased serum lysine, alpha-aminoadipic acid and alanine. During normal growth, dietary lysine is rapidly transported into muscle tissue, and concentrated in the intracellular space, forming a lysine reservoir [27]. As the first limiting amino acid for muscle accretion and growth in pigs, increased free lysine in serum suggests less incorporation into this reservoir or utilized for

protein synthesis. Further, alpha-aminoadipic acid is generated from lysine oxidation and is considered a strong indicator of tissue breakdown and turnover [28]. Considered together with the differences in growth performance between diets, these suggest lower rates of muscle accretion in CON and HF pigs [27]. The elevated concentration of alanine in serum of low protein fed pigs may also be indicative of this pattern. Most alanine is bound in muscle protein but can be utilized by the liver in the glucose-alanine cycle, particularly in response to inflammation or starvation [29]. Metabolic pathway analysis of HP vs. CON animals further supports these differences in tissue metabolism, highlighting key pathways found to be upregulated in HP-fed animals related to nitrogen balance and amino acid metabolism.

The presence of increased spermidine in serum of HP animals demonstrates that increased protein fermentation by gut microbes did occur in response to high-protein without added fibre [30]. This is further supported by the increased amino acid derivatives methionine sulfoxide, dimethyl arginine, and acetyl-ornithine observed in serum of both HP and HFHP diets. Increased methionine sulfoxide was also observed in the ileum of HP and HFHP animals and is indicative of increased methionine catabolism. Methionine sulfoxide is produced through the oxidation of methionine both in the mitochondria and by microbes [31,32]. While HP and HFHP diets did contain higher crude methionine (0.46% vs. 0.49% in low vs. high-protein diets respectively), the amount of methionine provided/kg of diet was not different when standard ileal digestibility was considered. It is therefore more likely that this indicates increased utilization of methionine by microbes, especially when considered alongside alterations to spermidine and other amino acid derived metabolites observed. This is further supported by difference in methionine catabolism identified during quantitative enrichment analysis comparing HP to CON diets (Figure 5.12).

The increased presence of both asymmetric and total dimethyl arginine in serum is indicative of increased arginine metabolism, a common metabolic pathway in microbes for growth and microbial protein synthesis [33]. Acetyl-ornithine is gut-microbiota derived, and a known intermediate in polyamine synthesis, correlated with spermidine abundance [34]. This set of metabolites is also well correlated with oxidative stress [35,36] and may be of particular interest for further investigation in the context of post-weaning diarrhea risk. Increases in valyrylcarnitine (C5) and beta-hydroxybutyrate are also of interest in this pattern as they may represent increased utilization of abundant leucine and valine in the HP and HFHP diets [37,38].

Differences in glycine, serine, arginine, and proline metabolism were also detected during QEA analysis of serum. This is in alignment with transcript differences suggestive of serine depletion in low protein diets. Glutamate is produced as part of the glycolytic pathway of serine production and was found to be increased in the CON diet. Also of note is the relationship between serine and sphingolipid production. Serine is required for sphingolipid and phospholipid synthesis [39,40], and there may be a relationship between the increased sphingolipids identified in CON- and HF-fed pigs and the alterations in serine metabolism both at the intestinal and systemic level. This increased phospholipid and sphingolipid production could drive serine depletion and resulting cecal tissue responses observed in CON and HF animals.

While increased trans-hydroxyproline was also observed in the ileum of CON pigs and may be an indicator for tissue catabolism [41,42], this compound is also abundant in fish meal [43]. Therefore, in this study it is likely that the difference observed in this metabolite is due to differences in dietary protein source. Despite this limitation, the pattern observed in lysine, arginine and alpha-aminoadipic acid suggests that the lower growth performance in pigs on the low protein diets is due to lower rates of muscle accretion and inadequate protein levels to support

optimal growth during this period. This is further supported by the lower glutamate observed in serum of pigs fed the high-protein diets, while other metabolites occurring further down the urea cycle pathway are increased in the same animals. This further suggests that HP and HFHP pigs were able to utilize increased dietary protein for amino-acid synthesis and tissue accretion.

Low-protein diets increase serine synthesis and cellular stress response

Of the seven differentially expressed genes detected in cecal tissue, four are members of the serine synthesis pathway or the related integrated stress response (ISR) pathway triggered in response to L-serine depletion. Serine and glycine are integral to cellular homeostasis, growth, and proliferation. They are important precursors for key cellular processes including synthesis of purine nucleotides, ATP production, folic acid metabolism, and glutathione production [44]. In conditions of serine depletion, cells can compensate through de novo L-serine synthesis from glucose via the PHGDH pathway. Two of the three key enzymes in this pathway, PSAT1 and PSPH, were found to be differentially expressed in pigs fed a high-protein diet. The expression of PSAT1 shows the largest protein-dependent difference with approximately 10-fold decreased expression in both high-protein diets compared to those with lower protein (Figure 5.14). While differences in PSPH were significant, but less pronounced. These two enzymes constitute the second and third steps of the serine synthesis pathway, where PSPH catalyzes the final production of serine to compensate for serine starvation [45]. These results suggest that in a low-protein post-weaning diet, serine starvation of intestinal tissue may occur.

Serine deprivation induces activating transcription factor 4 (ATF4), for which CHAC1 and ASNS are also targets [44,46]. The transcriptional regulation of ATF4 is a key component of cellular ISR, resulting in a cascade of cellular stress responses [47]. In this study CHAC1 and ASNS expression were increased in the LP and HF diets compared to HFHP and HP. This pattern

suggests that there may be increased cellular stress in the cecal tissue in low protein fed pigs during the weaning transition. CHAC1 is a pro-apoptotic factor, and its overexpression can be associated with glutathione degradation and exacerbated oxidative stress [44]. Previous work has shown that CHAC1 is highly upregulated in the jejunum and colon during post-weaning diarrhea in pigs [48] and is linked to apoptotic signalling and ER stress in the cecal tissue of chickens following a disease challenge [49]. In vitro studies have also shown a role of CHAC1 in mediating endoplasmic reticulum and oxidative stress as well as apoptosis [48,50]. These in vitro studies have also shown a link between CHAC1 expression and increased stress-related tight-junction leakage and inflammation in porcine intestinal epithelial cells [50].

CHAC1 and ASNS up-regulation can be rescued through increased availability of serine or glycine to cells [44]. Interestingly, glycine is conditionally essential in young pigs [51] and previous studies have shown that increased glycine availability to small intestinal cells decreases oxidative stress and apoptosis while increasing cell growth and protein synthesis [52]. The pattern of gene expression shown in this study is suggestive of the ATF4 induction of both PHGDH and ISR pathways due to low levels of serine availability. While dietary glycine may be an important factor in this response, no differences in serine hydroxymethyltransferase, which converts glycine to serine, were detected. This suggests that it is the availability of serine itself which may underlie the response seen to low-protein diets in this study. This could in-part be due to the increased phospholipids detected in digesta and serum of pigs fed low-protein diets (Figure 5.9b), which would increase demand for free serine for phospholipid synthesis [39]. This also could be the result of oxidative stress-related alterations in glycolytic pathways that promote serine synthesis for downstream glutathione production [53]. This could be of particular importance in the context of

weaned pigs that experience high degrees of gastrointestinal oxidative stress, dysbiosis, and low feed-intake in the period immediately following weaning [54].

Alterations in serine metabolism alter further stress responses and nutrient sensing

ABHD4 is a serine hydrolase [55], considered to have multiple roles in lipid metabolism and has been linked to metabolic disease [56]. The expression ABHD4, was found to be increased in both the high-protein diets (HP and HFHP) compared to HF and may be the result of alterations in serine availability suggested above, as serine is required in the enzyme's active site. ABHD4 is a key regulator of N-acyl phospholipids in the central nervous system [57] including N-acylphosphatidylethanolamine (NAPE), which are secreted from the small intestine in response to dietary fat [58], and therefore influencing satiety and subsequent dietary intake. The presence of increased ABHD4 in the cecum of HP and HPHF pigs suggests that increased protein feeding also has positive impacts on gut-brain axis signalling through maintaining the availability of serine for synthesis of ABHD4. Further, ABHD4 is important for the conversion of NAPE to arachidonyl-ethanolamide, an endocannabinoid associated with the regulation of inflammation [59].

ADM2, which was decreased in high protein-fed pigs, is also under ATF4 regulation and is associated both with response to cellular stress and with lipid homeostasis and anorectic signalling [60,61]. ADM2 is produced by intestinal epithelial cells in response to dietary-induced oxidative stress, and was also upregulated in colitis, helping to resolve inflammation [62]. ADM2 has roles in whole body metabolism and has been shown to improve insulin sensitivity and alter adipose tissue deposition through effects on adipocytes and M2 macrophages [63]. In the context of this study, decreased ADM2 may contribute to the improved markers of lean tissue deposition identified in the serum metabolome of HP and HFHP animals.

GPT2 contributes to glutathione production through generating glutamate and is important to cellular response to oxidative stress [64]. Similar to other altered transcripts detected in this study, it is under ATF4 regulation. GPT2 is a major aminotransferase known to regulate homeostasis and is involved in gluconeogenesis [65]. During periods of dietary restriction, GPT2 is produced from dietary alanine and used to support intestinal stem-cells [66]. Due to its important role in transamination, GPT2 has roles in arginine, glycine, and serine metabolism [67,68]. The role of GPT2 in gluconeogenesis is particularly interesting in the context of the weaned pig, especially when considered alongside alterations in glycolytic serine synthesis and the tissue catabolism differences. Plasma glucose was not significantly different between any groups, except for the HF diets which was slightly decreased compared to all others. However, it is unlikely that differences in glucose would be observed in these diets that meet dietary requirements for weaned pigs. Overall, these patterns suggest an important role of dietary protein in maintaining homeostatic functions of cecal tissue during the post-weaning period, with a pronounced impact on oxidative stress and cellular integrated stress response when low dietary protein is fed.

Increased dietary protein supports tissue function and growth post-weaning

Serine availability and metabolism was found to be a key component of the differences observed between pigs on high and low protein diets. Glycine and serine metabolism was shown to be enriched in HP versus CON pigs, in contrast to an increase in de novo serine synthesis and associated integrated stress response genes in pigs fed a low protein diet. This may in part be due to the increased phospholipid and sphingolipid secretion in both intestinal compartments of CON and HF animals. However, it is not possible within the limitations of this study to determine if this is a key driver of reduced serine availability in the cecum of animals on the low-protein diets. The

importance of serine to many physiological functions makes it an area of interest for further exploration.

Altered phospholipids and sphingolipids suggest increased bile acid secretion in low-protein diets

In contrast to the effects on microbial composition, protein was the main driver of differences in metabolites. In all three locations sampled, increased phosphatidylcholine (PC) species were observed in low-protein diets. The increased presence of many different PC species alongside increased lysophosphatidylcholine species, sphingomyelin, and taurine in the ileal digesta of pigs on lower protein diets is highly suggestive of increased bile secretion or bile acid deconjugation between high and low protein diets. Previous studies have shown that plasma PC composition is shaped by dietary protein type (Sugano et al., 1988). Plasma PC composition has been found to be altered in response to soybean vs. casein as a source of dietary protein, where methionine availability appears to be particularly important [69]. However, this study observed alterations in response to methionine supplementation that was 10 times greater than the difference in methionine content between the diets examined here. These effects on PC abundance in both the intestinal lumen and systemic circulation is of interest for further exploration due to the role of PCs in intestinal epithelium barrier function. PC equilibrium in intestinal epithelial cells is a key factor in intestinal homeostasis, and alterations quickly result in colitis in mouse models [70].

Due to technical limitations of earlier techniques, which have only recently been resolved through MS-based lipidomics, there is limited information in the literature about these compounds at the individual level [71]. Therefore, it was not possible to fully leverage the PC changes detected in this study in metabolic pathway analysis. The kit-based method utilized in this study was able to provide this more detailed lipid species level annotation on PCs observed in the intestinal digesta

and plasma in response to differing dietary protein levels which will inform future studies in weaned pigs.

Alterations in dietary fibre and protein impact cecal but not ileal microbiota

Decreased Shannon diversity observed in pigs fed both high-fibre diets is suggestive of better support of important and highly abundant microbial constituents when higher levels of fermentable carbohydrate are available. Higher α-diversity is often observed during the early post-weaning period due to the presence of an increased number of low-abundance incidental taxa (Diether et al., submitted). Previous work assessing susceptibility to post-weaning diarrhea has also found higher Shannon diversity is associated with diarrhea susceptibility [72].

The effect of dietary fibre and protein on cecal but not ileal microbiota composition is of particular interest given concerns around the role of protein in pathogen expansion and post-weaning diarrhea in weaned pigs. Low protein diets are thought to reduce post-weaning diarrhea risk through reducing the ability of pathogens, especially ETEC, to expand in the small intestine [73]. While there is strong evidence to support that these dietary changes are an effective management strategy for post-weaning diarrhea [3], most of the studies examining the linkages between protein fermentation and post-weaning diarrhea utilize diets which are also affected by differences in energy density, plant glycans and polyphenols, and anti-nutritional factors that result from altering the ratios of ingredients such as soybean meal and cereals. Comparatively, the differences in dietary protein and fibre obtained in this study were created through utilization of semi-purified diets, allowing for a focus on the effects of this increase in fibre or protein itself without any confounding effects of the differences listed above. From this perspective, the increase of dietary protein in weaned pig diets to a level more comparable to what is obtained from sows'

milk does not itself result in substantial changes to the ileal microbiota or expansion of any known pathobiont species.

While differences in cecal microbiota were observed in response to dietary changes, robust taxa-level differences were only observed for two genera *Butyricicoccus* and *Bulleida*. All ASVs in each of these genera were also assigned a species-level identification - *Butyricicoccus pullicaecorum* and *Bulleida* p-1630-c5 respectively. *Bulleida* is not well described within the pig gut microbiota but has been identified with increased abundance in the human gut microbiota in conditions associated with dysbiosis, including colorectal cancer, and type-2 diabetes mellitus [74,75]. This increase in abundance in HP pigs is mitigated with addition of dietary fibre, highlighting the role that fibre can play in mitigating the expansion of unwanted microbes when higher protein levels are fed. In contrast, *Butyricicoccus pullicaecorum* was increased in animals fed either diet containing additional dietary fibre compared to HP-fed animals. This butyrate producer has been shown to decrease lesions in a rat-model of IBD [76,77]. Originally isolated from broiler chickens, it has been successfully used as a probiotic in the same species, conferring resistance to intestinal pathogens, including *E. coli* and *Campylobacter* [78]. *B. pullicaecorum* has also been identified as a native constituent of the pig microbiota capable of conferring resistance to post-weaning diarrhea [79]. It is also of note that both taxa were detected with low relative abundance in the cecal microbiota. While relative abundance and relative functional contribution are not directly correlated, it is difficult to determine the relationship of these low-abundance taxa with functional changes in microbial metabolism within the limitations of this study. The lack of any changes in highly abundant taxa highlights the subtle degree of changes observed in response to these diets. The lack of difference in ileal microbiota diversity and the subtle shifts in

composition and genus-level differences of the cecal microbiota in response provide an avenue for further investigation of increasing dietary protein in weaned pig diets.

Through the results of this study, a clear effect of dietary protein on serum metabolome and cecal transcriptome was detected. The pattern of increased systemic lysine catabolism, reduced growth performance, and increased integrated stress response gene expression in intestinal tissue demonstrate that pigs fed a standard post-weaning diet may not be receiving sufficient dietary protein for optimal performance and intestinal tissue function. Overall, this indicates that increased dietary protein within a feasible range for commercial settings improves many indicators of tissue homeostasis and growth. While reduced dietary protein is thought to decrease the risk of pathogen proliferation and post-weaning diarrhea, alterations in the gut microbiota detected in this study were subtle. This suggests that with careful selection of ingredients, dietary protein could be increased to better support pigs during the post-weaning period without increased risk of post-weaning diarrhea.

5.5 References

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Chapter 6: General Discussion

The post-weaning period is a high-risk timepoint in pork production systems, with critical effects on long term pig health [1]. However, there is also great opportunity to improve animal health and decrease the need for therapeutic antibiotics through data-informed dietary strategies that reduce the incidence of post-weaning diarrhea (PWD). In this thesis, multi-omic tools were used to elucidate the mechanisms underlying benefits conferred by three common dietary interventions for weaned pigs. This research set out to identify alterations in gut microbiota, microbial metabolites, and host response resulting from three common dietary strategies for weaned pigs. These results will help to fill key knowledge gaps in our understanding of how these strategies are best integrated into weaned pig nutrition.

6.1 Benzoic acid and enzymes altered microbial metabolic networks

While much of the previous body of work examining dietary additives has focused on pathogen control and production of short chain-fatty acids, results from chapters 3 describes a more complex role for these additives in altering microbial metabolic networks.

In chapter 3, supplemented benzoic acid was identified in its undissociated form in the cecum of weaned pigs. This is an important finding as previous studies have shown rapid absorption and conjugation of benzoic acid after feeding [2,3]. A role for benzoic acid supplementation in increasing microbial cross-feeding products and decreasing products of valine degradation were identified as key changes in response to benzoic acid. These to findings are metabolically linked to alterations in propionate producers observed in microbiota analysis, as 1,2-propanediol production can support propionate production and reduce protein fermentation in the intestine [4-6].

Dietary enzymes contributed to improved fermentative capacity of the microbiota. Fibre fermenting families such as *Prevotellaceae* and members of order Bacteroidales were increased while genera with protein fermenting microbes such as *Selenomonas*, *Megasphaera*, and *Acidaminococcus* were decreased [7-9] These differentially abundant taxa suggest that xylanase within the enzyme blend fed may have a prominent role in microbiota effects [10,11]. Increased abundance of fermentable oligosaccharides resulting from fucose utilization provides a likely mechanism underlying the increased abundance of *Prevotellaceae,* which is an important indicator of resolution of post-weaning microbiota disruption [12]. The identification of 1-heptadecanol is also of interest given its correlation with Proteobacteria abundance, dysbiosis, and immunosuppression [13]. These results lend further support to the postulated prebiotic actions of feed enzymes, demonstrating increases in metabolites and microbial taxa associated with fibre-fermentation.

6.2 Medium-chain fatty acids support microbiota reassembly through bile acid secretion

The most pronounced shift in host-microbial metabolic networks was observed in response to the second additive blend explored in this thesis (MCOA). Improvements in indicators of whole-body metabolism 7- and 14-days post-weaning, alongside improved growth performance demonstrates an effect of MCOA on muscle accretion post-weaning. Reductions observed in creatinine, β-hydroxybutyrate, and L-carnitine further suggest that MCOA-fed pigs had reduced body fat mobilization and improved energy balance compared to control animals [14-16].

Perhaps the most important finding from this study was the potential relationship between improved metabolic status of MCOA-fed pigs and increased bile acid production and secretion. Increased cholic acid in plasma and colonic digesta alongside decreased plasma taurine and choline is highly suggestive of increased bile acid production and secretion [17,18]. Medium-chain fatty

acids (MCFAs) are specifically known to increase bile acid production compared to other dietary lipids [19]. In the context of the weaned pig, where bile acid production is inadequate [20,21], this effect of MCFAs may act to maintain bile acid production post-weaning. This bile acid abundance may also shape the post-weaning microbiota by conferring a competitive advantage to core intestinal microbes with bile acid tolerance mechanisms compared to incidental and opportunistic taxa that can proliferate following weaning [22]. This represents a key mechanism by which this additive blend may provide benefits after weaning.

6.3 Increased dietary protein supports muscle accretion and intestinal tissue homeostasis

The final experiment described in this work focused on using semi-purified diets to alter protein and fibre ratios in a highly controlled manner. Amplicon sequencing, metabolomics, and transcriptomics were employed to assess the impact of altering these ratios on gut microbiota, intestinal and host metabolome, and cecal transcriptome in detail.

Through this work, pronounced effects of dietary protein on the serum metabolome and cecal transcriptome were identified. Increases in markers of tissue breakdown and turnover were observed in pigs fed a standard post-weaning diet, including alpha-aminoadipic acid, and serum lysine and alanine[23,24]. At the transcriptomic level, pigs fed a standard post-weaning diet or a diet high in fibre showed increased expression of PSAT1 and PSPH, suggesting that low-protein diets result in serine starvation of intestinal tissue [25]. Increased expression of CHAC1 and ASNS further indicates that this serine-deprivation induces the cellular integrated stress response [26-28], and is highly correlated with oxidative stress [26]. While serine itself was not lower in the serum or digesta of pigs on the low protein diets, altered phospholipid and sphingolipid profiles in animals on the low protein diets may contribute to increased serine utilization [29,30], and therefore alter what is available to intestinal epithelial cells for first-pass metabolism.

These alterations in host metabolism and cellular stress response are important findings warranting further exploration, as the goal of reduced protein diets is to lower diarrhea risk without compromising performance [31], and suggest that current standards for reduced protein diets in weaned pigs have important implications for intestinal tissue function, amino acid metabolism, and lean tissue deposition. While the justification for low protein diets is to control pathogen proliferation risk, the use of highly controlled diets showed that increasing dietary protein to 23% resulted in subtle shifts to the cecal microbiota and no significant differences to ileal microbiota. Indicators of protein fermentation were detected in the high protein diet. However, these effects were decreased through the addition of fibre at the same protein level, demonstrating that protein fermentation can be controlled through small additions in highly fermentable fibre during this high-risk period. While the ingredients used in this study were selected to control for factors such as plant polyphenols and anti-nutritional factors, this study challenges current practices for lowering dietary protein post-weaning and suggests that with careful consideration of ingredients, increased dietary protein would better support the growth and physiology of weaned pigs without substantial alterations in gut microbiota or increased risk of post-weaning diarrhea.

6.4 Key findings and shared themes for the dietary strategies explored

While the additives and strategies explored in this study were found to differ in their mechanisms of modulating diet-microbiota-host interactions, several shared patterns emerged. From the perspective of microbiota modulation, benzoic acid, enzymes, and the MCOA blend evaluated provided benefits through improving microbial succession and the establishment of important constituents of the post-weaning microbiota. This suggests that an important evaluation strategy for acidifers in weaned pigs should be their ability to support the establishment of microbial species and metabolic networks that are known hallmarks of a mature and stable

microbiota, rather than focusing solely on pathogen exclusion. This focus on the patterns and characteristics of the growing pig microbiota can also be extended to interpretation of measures of α- and β-diversity. While increased α-diversity has traditionally been considered a positive indicator, in all three studies conducted here, increased α-diversity was associated with the presence of low-abundance, incidental taxa and not indicative of a microbiota rich in commensal species. Therefore, there is a need to conduct studies of these dietary strategies with appropriate baselines to compare. As shown in the MCOA study, collection of samples at 14 or even 21 days post-weaning allows for more appropriate assessment of effects on microbiota diversity and establishment by providing a stable microbiota as a basis of comparison.

Evaluation of host metabolome and transcriptome within these studies also provided key insights into future directions for exploration. Potential alterations in bile production and secretion were identified in response to both MCOA and alterations in dietary protein. Given the bactericidal effects of bile acids, and their important roles in shaping the gut microbiota, this is an interesting avenue for future exploration. The correlation between abundance of *Lactobacillus* species in the small intestine and increased tryptophan metabolite abundance in systemic circulation is also of interest. This challenges perspectives that impactful protein fermentation occurs solely in the large intestine [32], and may represent a key difference between the swine and human microbiota which is not yet well understood. Finally, the impact of reduced dietary protein on increased cellular stress in intestinal tissue stands in stark contrast to the benefits to gastrointestinal health typically thought to be associated with low protein diets [33]. These findings highlight the usefulness of multi-omic techniques to identify previously undescribed mechanisms for these dietary strategies and provide key information to inform further studies that will help move our understanding of

these mechanisms beyond correlations and improve our understanding of how to best support weaned pigs without the use of in-feed antibiotics.

6.5 Future Directions

Two key findings for the future implementation of dietary strategies for weaned pigs resulted from the work in chapters 3 and 4, identifying potential mechanisms for future exploration of these dietary strategies.

Future studies should focus on using quantitative methods to identify physiologically relevant thresholds for bile acid changes that result in benefits. A focus on identifying tryptophan metabolite concentrations in digesta through targeted metabolomics would also help resolve questions about the role of small-intestinal microbes on tryptophan metabolite production in the pig. Tryptophan metabolism is a hallmark of the mature pig microbiota, however our current understanding of microbial species contributing to this effect is limited.

While the results in chapter 3 provide new avenues for additive development and improvement, findings in chapter 4 challenge current practices in weaned pig nutrition, demonstrating important physiological changes in response to lowering dietary protein post-weaning. The use of semi-purified diets and molecular indicators of physiological change identified a detrimental effect of low-protein diets on host metabolome and transcriptome. However, additional work is needed to provide a complete picture of the effects of low-protein diets. Key limitations of this work include the need for a week-long adaptation period to metabolic pens, differences in animal- versus plant-based protein sources, and limited information about changes in the small-intestinal or host transcriptome. Follow up work will therefore be needed in three phases. The first would be addition of bile acid metabolomics, small-intestinal transcriptomics and metagenomic sequencing to a similar experiment to more wholistically

understand the changes in holobiont metabolism occurring. Integration of these more complete metabolic and microbial networks will be key in understanding the role of protein fermentation versus synthesis in the results seen, and the degree to which this affects intestinal function. Following this, translation into commercially relevant conditions is needed. The work described in chapter 4 was completed in a high-health status facility in non-standard housing. Therefore, follow-up work should take place with an *E. coli* challenge before any recommendations can be made. Finally, dependant on results of challenge work, translation of these findings into commercially relevant diets would be needed.

The use of multi-omics techniques in this thesis demonstrates the power of utilizing these increasingly accessible methods to further our understanding of current nutritional strategies. The ability to examine diet-microbiota-host interactions with improved resolution is an impactful tool to better understand the application of current strategies and inform directions for improvement. Through this work, it is possible to identify key changes in physiology and metabolism that occur during after and use targeted, data-informed dietary strategies to better support a healthy and expedient weaning transition.

6.6 References

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Appendix A: Supplemental Tables and Figures

Table A1.Compounds identified by feature selection as differing between the benzoic acid and control diets. For identified compounds, library match scores, retention index match (Ri_{lib}-Ri_{exp}) and CAS identification number are provided.

Metabolite Name	Count	Average Area	Quant Mass	Library Match	Retention Time 1, s	Retention Time 2, s	$\lvert Ri_{Lib}-Ri_{exp}\rvert$	CAS
Analyte 4788	26	142566	268		1642	0.349		
1,2-Propanediol	26	152089	117	881	332	1.181	10.6	17887-27-3
Ethanol, (2-(3,4-dihydroxyphenyl)	50	56529	267	814	1024	1.092	28.9	68595-80-2
Benzoic Acid	50	4706820	179	952	614	0.059	9.9	2078-12-8
Analyte 1798	49	56981	265	650	872	1.344	10.4	51642-61-6
Analyte 2339	17	12719	232		1000	1.038		
Analyte 3231	22	12451	281		1173	1.440		
Analyte 1345	37	119979	174		762	1.870		
Analyte 164	38	8780	159		368	1.398		
Analyte 1210	40	20418	83		722	1.302		
Analyte 260	12	7128	145		413	1.134		
Analyte 3924	37	167370	129		1352	1.124		
Analyte 5000	30	14773	298		1745	1.215		
Analyte 4578	40	32541	399		1557	0.323		
Analyte 156	28	24762	159		360	1.444		
Analyte 2576	22	3945	369		1046	1.471		

Table A2. Compounds identified by feature selection as differing between the enzyme and control diets. For identified compounds, library match scores, retention index match (Ri_{lib}-Ri_{exp}) and CAS identification number are provided.

Metabolite Name	Count	Average Area	Quant Mass	Library Match	Retention Time 1, s	Retention Time 2, s	$\lvert Ri_{Lib}-Ri_{exp}\rvert$	CAS
Analyte 1293	48	29623	242		749	1.701		
Analyte 4496	43	454726	98		1530	0.131		
Analyte 1837	8	55540	268		888	1.443		
Analyte 3504	49	43152	173		1236	1.540		
Analyte 1646	23	7340	158		846	1.433		
Analyte 1654	12	41822	245		848	1.100		
1-Heptadecanol	34	291678	313	807	1196	0.990	3.9	144363-02-0
Analyte 3856	37	81625	222		1329	1.008		
Analyte 4276	20	33965	174		1453	1.064		
Glycine	45	6765840	86	805	674	1.147	3.9	5630-82-0
Analyte 4199	50	254340	281		1432	1.628		
Phenylalanine	34	1328029	218	899	931	1.396	8.7	2899-52-7
Analyte 4512	42	6751	444		1531	1.444		
L-(-)-Fucose	46	4837278	117	889	1008	0.974	45	n/a
Analyte 1087	15	18684	251		690	1.760		
Analyte 1598	16	18114	82		835	1.282		
Analyte 1926	19	20402	241		913	0.324		
Analyte 320	21	7128	145		434	1.107		
Analyte 1168	14	9157	258		708	1.695		
Analyte 2122	36	10582	271		950	0.373		
Analyte 5560	31	40881	58		2496	0.835		

Table A3. Calculated nutrient values for experimental diets. Diets were formulated with semi-purified ingredients to increase fibre (HF and HFHP) as well as protein (HP and HFHP).

To be provided per Kg of diet	CON	HF	HP	HFHP
gSIDlys/Mcal NE	6.13	6.12	6.12	6.13
Net Energy Mcal	2364	2393	2380	2317
CP %	18.13	18.91	24.30	24.94
Arg %	0.95	0.95	1.33	1.33
His %	0.40	0.40	0.61	0.61
Iso %	0.64	0.64	1.14	1.14
Leu %	0.40	0.40	0.61	0.61
Lys %	1.56	1.58	1.66	1.62
Met %	0.46	0.46	0.49	0.49
Cys %	0.27	0.27	0.41	0.41
Sul %	0.73	0.73	0.90	0.90
Phe %	0.74	0.74	1.32	1.32
Tyr %	0.48	0.48	1.03	1.03
Aro %	1.22	1.22	2.36	2.36
Thr %	0.40	0.40	0.61	0.61
Try %	0.28	0.28	0.33	0.33
Val %	0.73	0.73	1.32	1.32
Fat %	5.84	5.87	5.53	5.46
Fibre %	6.27	9.42	6.37	9.52
Ca %	0.85	0.85	0.85	0.85
Choline mg	849.67	849.67	849.67	849.67

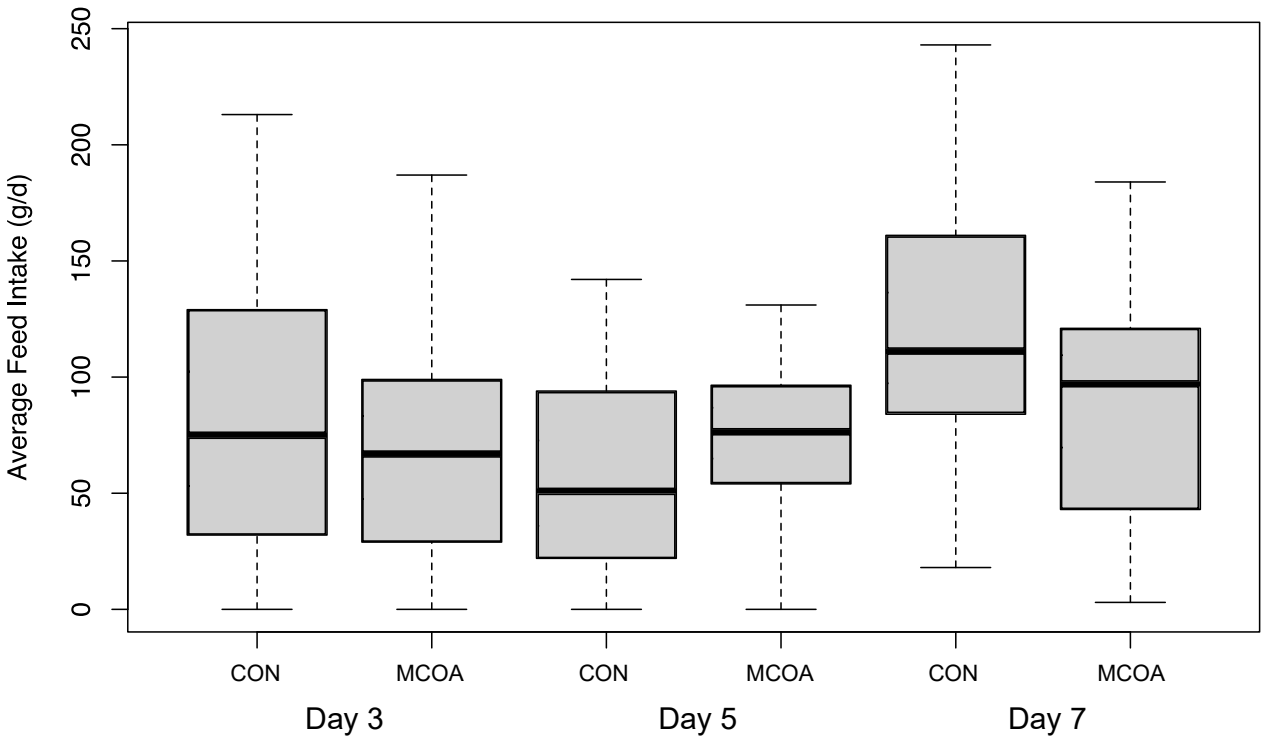

Figure A1. Comparison of daily feed intake of pigs from Day 3-7 post-weaning for pigs fed a control diet (CON) or a control diet supplemented with blend of medium chain fatty acids and organic acids on top (MCOA). Group names indicate treatment and day of sampling (7 or 14).

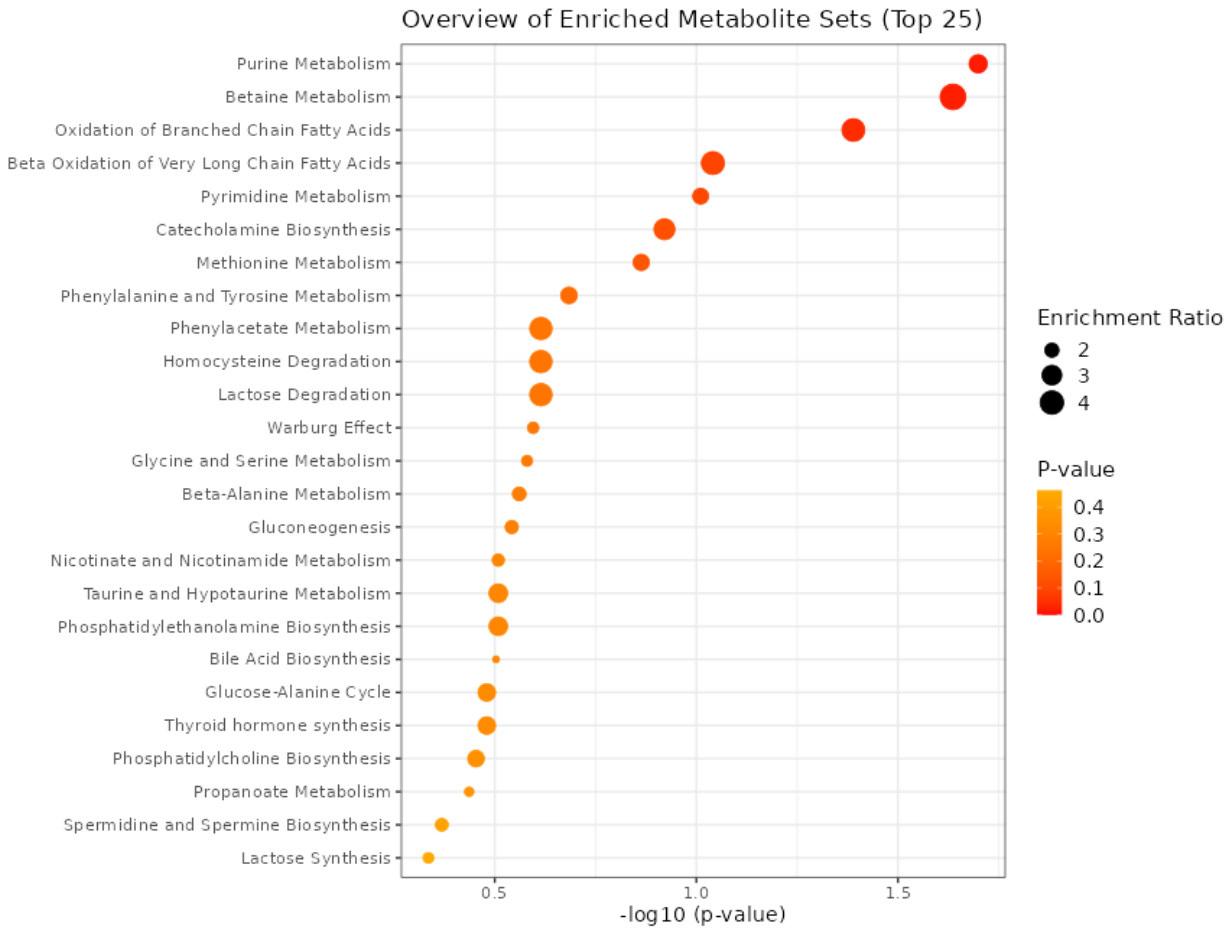

Figure A2.Metabolite pathways enriched in jugular plasma of CON pigs on D7 post-weaning.

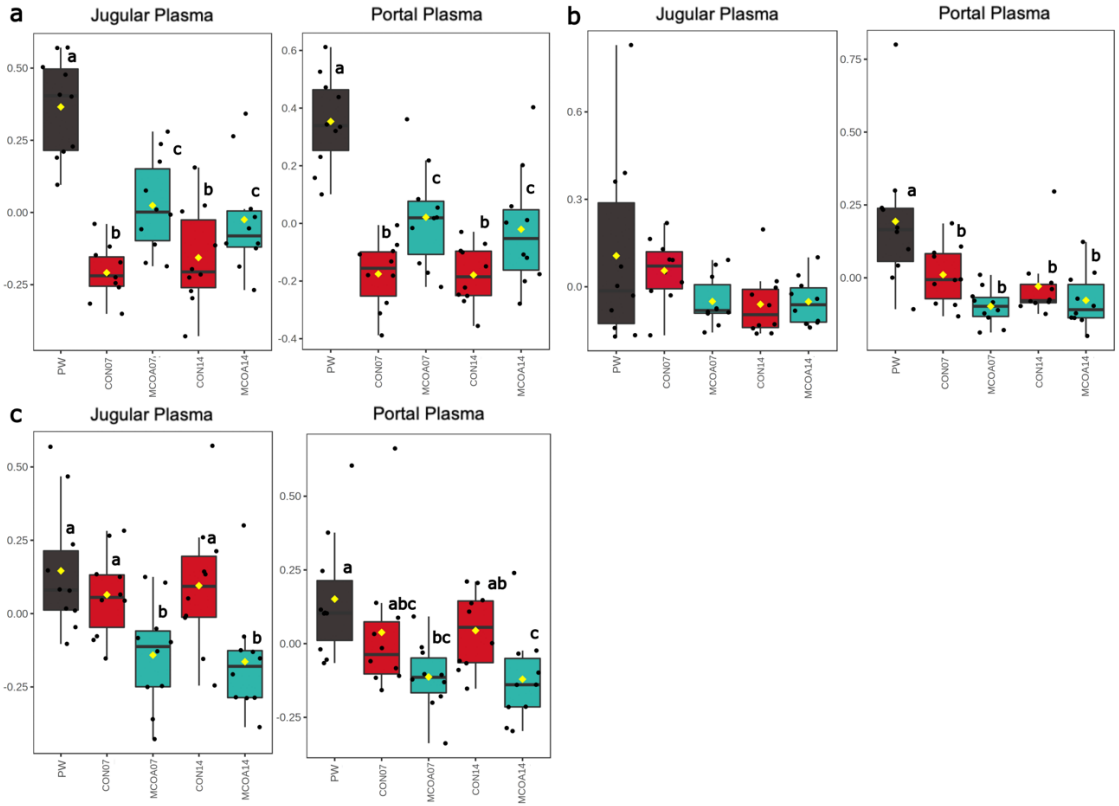

Figure A3. Differences in b-vitamins in response to a control diet or a diet containing MCOA: a. riboflavin, b. nicotinamide, c. pantothenic acid. Metabolomics analysis was performed using LC-MS/MS and a SWATH peak identification method. Statistical analysis was performed in Metaboanalyst 5.0. Compounds shown were not identified in intestinal metabolomics. Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Pre-weaning samples are denoted as PW. Groups with statistically different means ($P < 0.05$) are denoted by different letters.

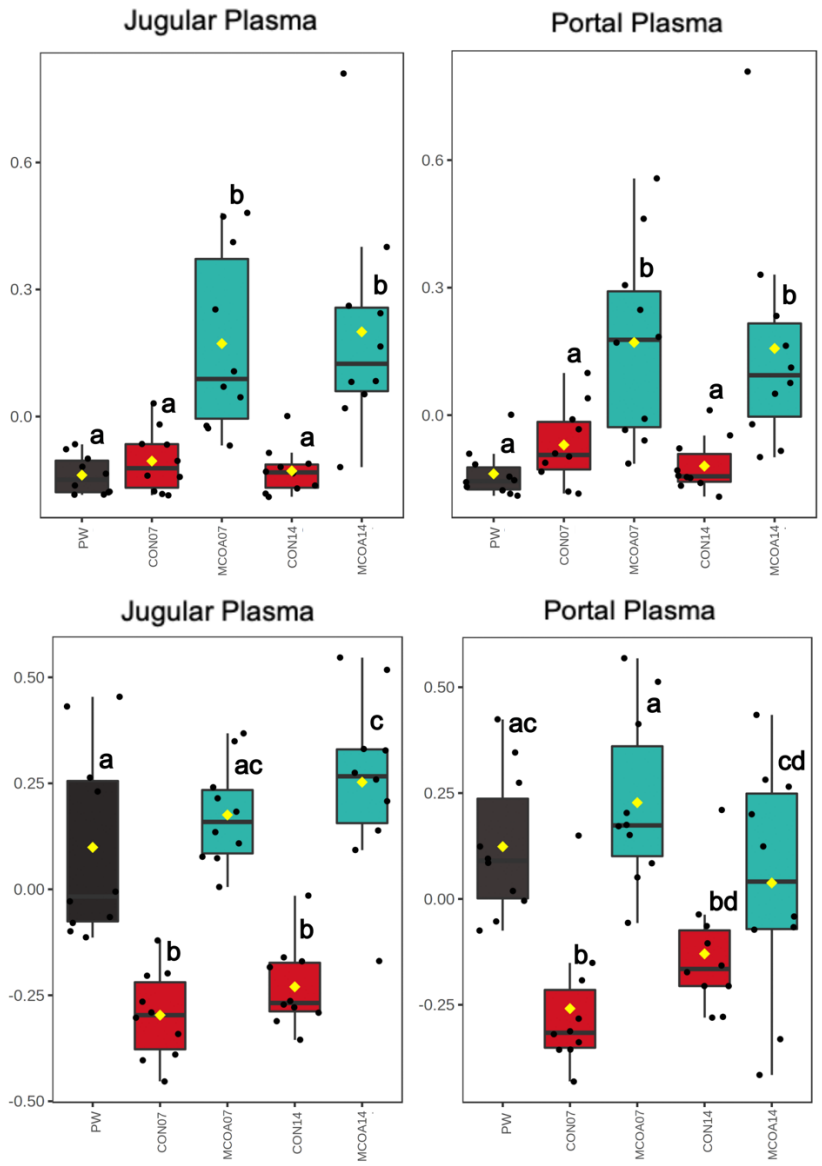

Figure A4. Additional tryptophan metabolite alterations in response to a control diet or a diet containing MCOA: a. 3-methyloxyindole, b. 2-methyloxyindole. Metabolomics analysis was performed using LC-MS/MS and a SWATH peak identification method. Statistical analysis was performed in Metaboanalyst 5.0. Compounds shown were not identified in intestinal metabolomics. Group names indicate treatment (CON vs. MCOA) and day of sampling (7 or 14). Pre-weaning samples are denoted as PW. Groups with statistically different means ($P < 0.05$) are denoted by different letters.