

Postbiotic strategies alleviate negative effects of subacute ruminal acidosis (SARA) on digestive and immune health in lactating dairy cows

by

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ABSTRACT

High-energy diets feeding in dairy farms can impact the homeostasis of gastrointestinal tract via changing the ecology of gut microbiota and their metabolites thus affecting the functionality of gut epithelium and host immune response, as a consequence, induce metabolic disorders in the body such as subacute ruminal acidosis (SARA). These disorders cause the changes in the fatty acid profile of the milk, affecting the welfare of dairy cows by increasing the incidence of infectious diseases such as systemic inflammation. Therefore, it is critical to find efficient solutions that optimize animal production without compromising gut health. It has been reported that *Saccharomyces cerevisiae* fermentation products (SCFP) as one of the postbiotics benefits rumen fermentation and overall performance in lactating dairy cows. However, cows respond differently to SCFP among studies. We speculate that this might be due to the fact that the mode of action of SCFP is not completely understood and as such the recommended dose of SCFP for each animal is not optimized.

In this study, we conducted two grain-based SARA challenges in early lactation of dairy cows. We found grain-based SARA challenges increases the lipopolysaccharides (LPS) concentrations in the rumen and hindgut, the concentrations of acute phase proteins and pro-inflammatory cytokines in peripheral blood. Grain-based SARA reduces the richness and diversity of rumen liquid and solid microbiota, alters its taxonomic compositions and predicted carbohydrates and nitrogen metabolic pathways. Besides, grain-based SARA reduces the number of significant associations among different taxa, the diversity of hub taxa in microbial co-occurrence networks in rumen liquid and solid

microbiota. We also investigated the possible benefit of two different SCFP (XPC, NutriTek) and the doses effect of NutriTek on immunity status and response, composition and functionality of microbial communities in the rumen and on to grain-based SARA challenges in lactating dairy cows. Our results showed that NutriTek with higher dose (38 g/d) helps to reduce pro-inflammatory status, improve the diversity and richness of the rumen liquid microbiota, promote the growth of cellulolytic bacteria in both rumen liquid and solid microbial communities, hence, attenuating adverse digestive and inflammatory responses associated with grain-based SARA challenges.

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FOREWORD

This dissertation is written in manuscript style and is composed of three manuscripts. The first manuscript has been accepted in the Journal of Dairy Science. The second manuscript is under review in the Journal of Dairy Science. The third manuscript have not yet been submitted to a journal. The titles and list of contributing authors of these manuscripts are as follows:

Manuscript 1: *Saccharomyces cerevisiae* fermentation products reduce bacterial endotoxin concentrations and inflammation during grain-based subacute ruminal acidosis in lactating dairy cows.

Contributing authors: J. Guo, L. Xu, H. Khalouei, K. Fehr, V. Senaratne, J. E. Ghia, I. Yoon, E. Khafipour and J. C. Plaizier.

Manuscript 2: Postbiotics from *Saccharomyces cerevisiae* fermentation stabilize microbiota in rumen liquid digesta during grain-based subacute ruminal acidosis (SARA) in lactating dairy cows.

Contributing authors: J. Guo, Z. Zhang, L.L. Guan, M. Zhou, I. Yoon, E. Khafipour and J. C. Plaizier.

Manuscript 3: Postbiotics from *Saccharomyces cerevisiae* fermentation stabilize rumen solid microbiota during grain-based subacute ruminal acidosis (SARA) challenges in lactating dairy cows.

Contributing authors: J. Guo, Z. Zhang, L.L. Guan, M. Zhou, I. Yoon, E. Khafipour and J. C. Plaizier.

CONTRIBUTION OF AUTHORS

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Manuscript 1: Dr. Kees Plaizier and Dr. Ehsan Khafipour did the funding acquisition and project administration. Dr. Kees Plaizier, Dr. Ehsan Khafipour and Junfei Guo designed this study. Junfei Guo, Hamid Khalouei, Kelsey Fehr and Vidura Senaratne did the animal trail, Junfei Guo and Lei Xu performed the lab work and data analysis. Junfei Guo drafted the manuscript. Junfei Guo, Dr. Kees Plaizier, Dr. Ehsan Khafipour, Dr. Le Luo Guan, Dr. Jean-Eric Ghia reviewed and edited the manuscript. Dr. Ilkyu Yoon did the funding acquisition, reviewed and edited the manuscript.

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Zhengxiao Zhang analyzed the data. Junfei Guo drafted the manuscript. Junfei Guo, Dr. Kees Plaizier, Dr. Ehsan Khafipour, Dr. Le Luo Guan, Dr. Jean-Eric Ghia reviewed and edited the manuscript. Dr. Ilkyu Yoon did the funding acquisition, reviewed and edited the manuscript.

Table of Contents

ABSTRACT	1
ACKNOWLEDGEMENTS.....	3
FOREWORD.....	4
CONTRIBUTION OF AUTHORS.....	5
LIST OF TABLES	10
LIST OF FIGURES.....	13
LIST OF ABBREVIATIONS	15
CHAPTER ONE	17
1.1 GENERAL INTRODUCTION	17
1.2 HYPOTHESES	22
1.3 OBJECTIVES.....	22
CHAPTER TWO.....	23
LITTERATURE REVIEW.....	23
2.1 GUT HEALTH IN RUMINANTS	23
2.1.1 Health of epithelia and immune status.....	24
2.1.2 Health of digesta.....	26
2.1.3. Gut microbiota in ruminants.....	28
2.2.3 Alternative techniques in gut microbiome study.....	37
2.3 ROLE OF DIET IN MODULATION OF GUT MICROBIOTA AND GUT HEALTH.....	41
2.3.1 High-grain diet feeding and gut health.....	41
2.3.2 The approaches for optimizing gut microbiota	53
2.4 SUMMARY.....	58
CHAPTER THREE.....	60
MANUSCRIPT 1	60
<i>Saccharomyces Cerevisiae</i> Fermentation Products Reduce Bacterial Endotoxin Concentrations and Inflammation during Grain-Based Subacute Ruminal Acidosis in Lactating Dairy Cows.....	60
3.1 ABSTRACT	60
3.2 INTRODUCTION	62
3.3 MATERIALS AND METHODS	64
3.3.1 Animals and diets	65
3.3.2 Experimental design.....	65
3.3.3 Sample collection and analyses.....	66
3.3.4 Statistical analysis.....	71
3.4 RESULTS.....	73
3.4.1 Effects of SARA on bacterial endotoxin (LPS and LTA) concentrations in the rumen, feces, and plasma.....	73
3.4.2 Effects of SCFP on bacterial endotoxin (LPS and LTA) concentrations in the rumen, feces, and plasma.....	76
3.4.3 Effects of SARA on inflammatory responses.....	76
3.4.4 Effects of SCFP on inflammatory responses.....	77
3.5 DISCUSSION.....	80
3.5.1 Assessment of SARA Induction Protocol.....	80
3.5.2 Effect of SARA on LPS concentrations in rumen fluid and feces	81

3.5.3 Effect of SCFP on LPS concentrations in rumen fluid and feces.....	82
3.5.4 Effects of SARA and SCFP on LPS and LBP concentrations in peripheral blood plasma.....	83
3.5.5 Effects of SARA and SCFP on LTA concentrations in peripheral blood plasma.....	84
3.5.6 Effects of SARA and SCFP on proinflammatory cytokines (IL1- β and IL-6) and APP (SAA and Hp) concentrations in peripheral blood plasma.....	85
3.6 CONCLUSIONS	87
CHAPTER FOUR	97
MANUSCRIPT 2	97
Postbiotics from <i>Saccharomyces Cerevisiae</i> Fermentation Stabilize Microbiota in Rumen Liquid Digesta during Grain-based Subacute Ruminal Acidosis (SARA) in Lactating Dairy Cows	97
4.1 ABSTRACT	97
4.2 INTRODUCTION	98
4.3 MATERIALS AND METHODS	102
4.3.1 Animals, Diet and Experimental design	102
4.3.2 Statistical Analysis	105
4.4 RESULTS.....	108
4.4.1 Alpha- and beta-diversity dynamics of rumen liquid microbiota.....	108
4.4.2 Compositional dynamics of rumen liquid microbiota in response to SARA challenges.....	111
4.4.3 Predicted functionality of rumen liquid microbiota.....	121
4.4.4 Association of SCFP supplementation and SARA challenges with Co-occurrence patterns of rumen liquid microbiota	122
4.4.5 Correlation between hub Taxa, biodiversity metrics of rumen liquid microbiota and rumen fermentation characteristics	126
4.5 DISCUSSION.....	128
4.5.1 Effects of grain-Based SARA challenges on diversity, composition and predicted functionality of rumen liquid microbiota.....	128
4.5.2 Effects of SCFP supplementation on diversity, composition and predicted functionality of rumen liquid microbiota	134
4.6 CONCLUSIONS	138
CHAPTER FIVE	140
MANUSCRIPT THREE.....	140
Postbiotics from <i>Saccharomyces Cerevisiae</i> Fermentation Stabilize Rumen Solid Microbiota during Grain-based Subacute Ruminal Acidosis (SARA) Challenges in Lactating Dairy Cows.....	140
5.1 ABSTRACT	140
5.2 INTRODUCTION	142
5.3 MATERIALS AND METHODS	145
5.3.1 Animals, diet and experimental design.....	145
5.3.2 Statistical analysis.....	146
5.4 RESULTS.....	150
5.4.1 Alpha- and beta-diversity dynamics of rumen Solid microbiota	150
5.4.2 Compositional dynamics of rumen solid microbiota	151
5.4.3 Predicted functionality of rumen solid microbiota.....	160
5.4.4 Effect of SCFP supplementation and SARA inductions on co-occurrence patterns of rumen solid microbiota.....	161
5.4.5 Correlation between hub taxa, biodiversity metrics of rumen solid microbiota and rumen fermentation characteristics	167
5.5 DISCUSSION.....	176
5.5.1 Effects of Grain-based SARA challenges on diversity, composition and predicted functionality of rumen solid microbiota.....	176
5.5.2 Effects of SCFP supplementation on diversity, composition and predicted functionality of rumen solid microbiota.....	182

5.6 CONCLUSIONS	187
CHAPTER SIX	188
GENERAL DISCUSSION.....	188
6.1 GENERAL DISCUSSION	188
6.2 LIMITATIONS	196
6.3 FUTURE WORK	197
CHAPTER SEVEN	198
CONCLUSIONS	198
REFERENCES.....	200

LIST OF TABLES

Table 3-1. Ingredients and chemical composition of experimental diets.....	67
Table 3-2. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) and parity on rumen and feces free lipopolysaccharide (LPS) concentrations of lactating dairy cows.....	74
Table 3-3. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1.1, and SARA1.2) and parity on lipoteichoic acid (LTA) concentration in blood plasma of lactating dairy cows.....	75
Table 3-4. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) and parity on peripheral blood acute phase proteins and cytokines concentrations of lactating dairy cows.....	78
Table 3-5. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and sampling periods (Pre-SARA1, Post-SARA2) on myeloperoxidase (MPO) concentrations (ng/μg total protein) in rumen papillae of lactating dairy cows.....	79
Table 3-6. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on rumen and feces free lipopolysaccharide (LPS) concentrations of lactating dairy cows.....	90
Table 3-7. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on rumen and feces free lipopolysaccharide (LPS) concentrations in parity 2 and 3 lactating dairy cows.....	91
Table 3-8. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1.1, and SARA1.2) as well as parity on lipoteichoic acid (LTA) concentration in blood plasma of lactating dairy cows.....	92

Table 3-9. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) and parity on blood acute phase proteins and cytokines of lactating dairy cows.....	93
Table 3-10. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on lipopolysaccharide (LPS) binding protein (LBP) in different parities.....	94
Table 3-11. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on interleukin-1 β (IL-1 β) in different parities.....	95
Table 3-12. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on interleukin-6 (IL-6) in different parities.....	96
Table 4-1. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (from 4w before until 12 wk after calving) on alpha-diversity of rumen liquid microbiome in lactating dairy cows.....	114
Table 4-2. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on the relative abundances (%) of the main phylum of rumen liquid microbiome in lactating dairy cows.....	116
Table 5-1. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (from 4 wk before until 12 wk after parturition) on alpha-diversity of rumen solid microbiome in lactating dairy cows.....	154
Table 5-2. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on the relative abundances (%) of the	

main phylum of rumen solid microbiome in lactating dairy cows.....	156
Table 5-3. The comparison of network properties in each treatment group	
(Control, SCFPa, SCFPb-1X, and SCFPb-2X) between non-SARA and SARA	
stages.....	166

LIST OF FIGURES

Figure 2-1. Ruminal microbiota and metabolic diseases in ruminants.....	31
Figure 2-2. Proposed mechanisms through which high grain feeding causes inflammation in dairy cows.....	46
Figure 3-1. Experimental design.....	68
Figure 4-1. nMDS of Bray-Curtis distances of rumen liquid microbial communities.....	110
Figure 4-2. Longitudinal shifts in Firmicutes and Bacteroidetes proportions in rumen liquid microbiota.....	112
Figure 4-3. Differentially abundant taxa in rumen liquid microbiota in control vs. SCFPb-2X.....	117
Figure 4-4. Differentially abundant taxa in rumen liquid microbiota in control vs. SCFPb-1X.....	118
Figure 4-5. Differentially abundant taxa in rumen liquid microbiota in control vs. SCFPa.	119
Figure 4-6. Differences in predicted functions of rumen liquid microbiota between control and SCFPb-1X and 2X groups.....	120
Figure 4-7. Microbial interaction networks.....	123
Figure 4-8. Distribution of hub taxa within each treatment group during non-SARA and SARA stages.	127
Figure 4-9. Correlation among hub taxa and rumen fermentation characteristics and biodiversity metrics of rumen liquid microbiota.....	130
Figure 5-1. nMDS of Bray-Curtis distances of rumen solid microbial communities among treatments (A) and stages (B).	157
Figure 5-2. Longitudinal shifts in Firmicutes and Bacteroidetes proportions in rumen solid microbiota.....	158
Figure 5-3. Longitudinal shifts in Proteobacteria and Fibrobacteres proportions in rumen solid microbiota.	159
Figure 5-4. Differentially abundant taxa in rumen solid microbiota in control vs. SCFPb-2X.	168

Figure 5-5. Differentially abundant taxa in rumen solid microbiota in control vs. SCFPb-1X.	169
Figure 5-6. Differentially abundant taxa in rumen solid microbiota in control vs. SCFPa.	170
Figure 5-7. Differences in predicted functions of rumen solid microbiota between control and SCFP groups during SARA.	171
Figure 5-8. Differences in predicted functions of rumen solid microbiota between control and SCFPa groups during SARA.	172
Figure 5-9. Microbial interaction networks in rumen solid digesta.....	173
Figure 5-10. Distribution of hub taxa within each treatment group during non-SARA and SARA stages.	174
Figure 5-11. Correlation among hub taxa and rumen fermentation characteristics and biodiversity metrics of rumen solid microbiota.	175

LIST OF ABBREVIATIONS

ADF = Acid detergent fiber

ANOVA = Analysis of variance

APP = Acute phase protein

ASV = Amplicon sequence variants

CAZyme = Carbohydrate active enzyme gene families

CoNet = Correlation network analysis

CP = Crude protein

CSS = Cumulative sum scaling

DFM = Direct-fed microbes

DGGE = denaturing gradient gel electrophoresis

DM = Dry matter

DMI = Dry matter intake

eNDF = Effective neutral detergent fiber

FDR = False discovery rate

GIT = Gastrointestinal tract

Hp = Haptoglobin

ISAPP = International scientific association for prebiotics and probiotics

LBP = LPS-binding protein

TLR = Toll-like receptor

LPS = Lipopolysaccharide

LTA = Lipoteichoic acid

MetaLonDA = Metagenomic longitudinal differential abundance

MPO = Myeloperoxidase

NDF = Neutral detergent fiber

NetCoMi = Network Construction and comparison for Microbiome data

NF- κ B = nuclear factor κ B

NGS = next-generation sequencing

nMDS = Non-metric multidimensional scaling

PCoA = Principal coordinate analysis

PCR = Polymerase chain reaction

PERMANOVA = Permutational multivariate analysis of variance

PICRUSt = Phylogenetic investigation of communities by reconstruction of unobserved states

RDP = Ribosomal Database Project

RFLP = restriction fragment length polymorphism

SAA = serum amyloid A

SARA = Subacute ruminal acidosis

SAS = Statistical analysis system

SCFA = Short-chain fatty acid

SCFP = *Saccharomyces cerevisiae* fermentation products

SEM = Standard error of means

TMR = Total mixed ration

TNF- α = tumor necrosis factor- α

VFA = Volatile fatty acids

CHAPTER ONE

1.1 General introduction

Healthy gastrointestinal tract contains barrier junction of epithelium as part of immune system to block the entry of infecting microbes, harbors a large amount of immune cells including lymphocytes, mast cells, macrophages and other cells in the gut epithelium that participate in both innate and adaptive immune responses during infections (Cummings et al., 2004; Belanche et al., 2021). There are large amounts of microbes in the gut that help to digest and absorb the nutrients, which produce enzymes involved in feed substrates degradation in the gastrointestinal tract (McCann et al., 2017). In ruminants, there are billions of microorganisms including bacteria, fungi, protozoa, archaea and bacteriophages in the rumen that play a crucial role in immunological, metabolic and physiological processes in the host. They live inside the rumen at a symbiotic relationship while maintaining the rumen homeostasis (Russell and Rychilik, 2001; Zhou et al., 2015; McGovern et al., 2018). Large intestine also contains microbes like rumen, and the enteric microorganisms become constant in weeks after birth. Intestinal microbiota can digest and absorb the rumen fermentation products and by-pass nutrients such as starch (Glock and DeGroot, 1998). Some nutrients that escape the rumen degradation or fermentation enter the small intestine and large intestine for further digestion to supply energy for the host. Starch fermentation in gut produces more energy than in the rumen (Gressley et al., 2011; Plaizier et al., 2017; Plaizier et al., 2018).

Rumen microbes can be influenced by diet changes. Microbes shift easily when the available substrates change in the rumen, members that can utilize the food most become

more abundant (Henderson et al., 2015, Zhou et al., 2015). Cellulolytic microbes are more abundant in high forage diet feeding condition, and amylolytic members increases after grains proportion increases (O'Hara et al., 2020). Rumen fermentation contributes the most in terms of microbial fermentation in GIT, is crucial to the growth and production of animals. Volatile fatty acids (VFA) mainly acetate, butyrate and propionate are the major products from rumen fermentation, and they provide around 70% of the energy requirements of the host (Bergman, 1990). Grain diet feeding increases the production of VFAs and lactic acid in the rumen. Large amount of VFAs cause acidity to increase in the rumen and may cause acidosis (Khafipour et al., 2009a; Khafipour et al., 2009b; Li et al., 2016). In normal conditions, gut microbial environment is in homeostasis which contains symbiotic, commensal and pathogenic species and they require a stable enteric condition for maintenance and growth. Low gut pH disrupts this homeostasis and some microbes that are tolerant to low pH can take the advantage and other members that are sensitive to low pH decrease such as lactic acid utilizing bacteria (Khafipour et al., 2009c; Petri et al., 2013; Zhou et al., 2015; Khafipour et al., 2016). High grain diet increases the production of ammonia, which can be converted to urea in the liver to increase the dietary nitrogen utilization. But large amounts of ammonia that exceeds the liver detoxification and accumulates in the lumen and become toxic (Wallace, 1979). During lactation or late pregnancy, ruminants also suffer a negative energy balance due to higher demand production of milk and fetal development. The oxaloacetate can preferentially be used for glucose synthesis and not available for acetyl-CoA in Krebs' cycle, and consequently this increases the production of ketone bodies which will be toxic to the body in large amounts (Schwab and Broderick, 2017). Meanwhile, low pH

disrupts the gut barrier function and increases the possibility of infectious microbes entering the lumen and thus, induce a local or systemic inflammation (Cummings et al., 2004).

Studies have demonstrated that subacute ruminal acidosis (SARA) is a common disease in high yield dairy cows that causes substantial economic loss to dairy farms (Krause and Oetzel, 2006; Plaizier et al., 2008; Plaizier et al., 2012). There are no visible signs that distinguish SARA from other diseases with common clinical signs of SARA being fluctuations in feed intake and reduced milk yield, however, it has also been associated with systemic inflammation along with liver abscesses, and lameness (Krause and Oetzel, 2006; Plaizier et al., 2008). SARA is defined by a moderate depression in rumen pH (Garrett et al., 1999) ranging from 6 to 5.2 (Li et al., 2013). This depression in pH is initiated by changes in substrates available for microbial fermentation in the rumen and hindgut as a result of a transition to a high grain diet or other increase in other highly fermentable carbohydrates. As a result, in early lactation, when cows are transitioned to a high-grain diet to compensate for a negative energy balance, cows are at risk for developing SARA (Krause and Oetzel, 2006; Oetzel, 2007). Changes in the substrates available in the rumen and hindgut can result in changes to rumen and hindgut microbial populations, changes to microbial metabolites, and subsequently changes to rumen conditions, such as a reduced rumen pH (Plaizier et al., 2018; Khalouei et al., 2020). High grain diet may result in microbial dysbiosis in the rumen and hindgut. This dysbiosis involves effectively large-scale changes in microbial populations, and with this, comes the disintegration of gram-negative bacterial groups unadapted to new rumen and hindgut conditions, and proliferation of those that prefer the new conditions (Plaizier et al., 2012;

Mao et al., 2013). This results in the release of lipopolysaccharides (LPS) that may translocate into the blood stream (Plaizier et al., 2008; Khafipour et al., 2009b; Li et al., 2012). This LPS translocation, or perhaps translocation of other immunogenic compounds released as a result of microbial dysbiosis, may cause systemic immune responses during this time (Li et al., 2016; Eckel and Ametaj, 2016). LPS can bind to toll-like receptors on cell surface and trigger the production of pro-inflammatory cytokines and acute phase proteins (APPs) (Eckel and Ametaj, 2016). Therefore, it is critical to find efficient solutions that optimize gut health during nutritional challenges in dairy cows. A number of manipulation strategies have been developed to attenuate the adverse effects of SARA. Feed additives are usually applied for maintaining microbiota and gut health, such as buffers, antibiotics, probiotics, prebiotics and postbiotics (Wegh et al., 2019; Michalak et al., 2021). In recent years, the high capacity of probiotics and prebiotics or the combination of them to provide beneficial health effects led to the growing scientific and commercial interests as a health promoting strategy (Retta et al., 2016; Petri et al., 2020). Probiotics are commonly used in ruminant production, and its effects have been shown on modifying microbial population, maintain rumen health and enhance animal production and performance (Michalak et al., 2021; Hadhoud et al., 2022). Nowadays, postbiotics draw more attention because of their stability and wide range of functionalities, and the microbial metabolites that contribute to communication between gut microbes, and microbes and the host (Wegh et al., 2019). For example, hydrolyzed yeast supplementation in calf starter diets improved the immune-related serum protein such as serum haptoglobin (Hp) production in neonatal calves after vaccine challenge (Kim et al., 2011). Yuan et al. (2015) also found that yeast products modulated uterine

inflammatory cytokines and enhance the humoral and mucosal immunity in cows during the transition period. However, mechanisms for the effects of yeast products on the immune system are unclear. It has also been reported that feeding yeast culture could modulate the systemic immune response during SARA through reducing the LPS concentration in the rumen during SARA (Li et al., 2012). *Saccharomyces cerevisiae* fermentation product (SCFP) has been widely used in ruminant nutrition as a rumen fermentation modifier. It has been reported that SCFP has benefits on rumen fermentation and overall performance in lactating dairy cows (Moya et al., 2009, Yalcin et al., 2011, Zhu et al., 2017). The stabilizing effect of SCFP on rumen fermentation may be due specifically to *S. cerevisiae* metabolites it contains, such as amino acids, organic acids and vitamins, that can be used as nutrients by cellulolytic bacteria and lactate-utilizing bacteria, which are important for rumen fermentation (Wiedmeier et al., 1987, Moya et al., 2009, Wagner et al., 2016). Metabolites within SCFP may also alter rumen pH as well as volatile fatty acids concentrations (Williams et al., 1991). Although several mechanisms of action for the effect of SCFP on rumen fermentation have been proposed, the exact mode of action is still not known.

In this study, we conducted two SARA challenges by increasing dietary starch content during early lactation in dairy cows. Except for Control group, cows in treatment groups were supplemented with two SCFPs (XPC and NutriTek). In order to investigate the dose effect, NutriTek was supplemented at two different doses. We aimed to find if these two SCFPs could reduce the endotoxin concentrations in the rumen, feces and peripheral blood, and reduce pro-inflammatory responses in dairy cows under SARA conditions. We also determined the rumen microbial population dynamics during SARA,

to investigate the effect of SCFP on stabilizing rumen microbial environment such as prevent the losses in abundance of fibrolytic bacteria when dietary starch increases, hence stabilizing rumen pH.

1.2 Hypotheses

1) Grain-based SARA challenges cause systemic inflammatory responses in dairy cows and rumen microbiome dysbiosis, including unfavourable changes in the composition and functionality of the rumen microbiome.

2) A repeated SARA challenge is more severe than a single SARA challenge.

3) SCFP reduces the inflammatory responses and stabilizes the rumen microbiome with a dose dependent effect during SARA challenges in dairy cows.

1.3 Objectives

To evaluate the effect of two commercially available postbiotics (XPC and NutriTek, Diamond V, IA, USA) on indicators of digestive and immune health including;

1) The endotoxins concentrations in the rumen, feces and blood circulation,

2) Proinflammatory responses in peripheral blood including concentrations of APPs and proinflammatory cytokines, and also inflammation in rumen papillae,

3) Microbial population dynamics and predicted functionality in rumen liquid and solid digesta in dairy cows subjected to subacute ruminal acidosis (SARA) challenge.

CHAPTER TWO

LITERATURE REVIEW

2.1 Gut health in ruminants

In recent years, the term ‘gut health’ has gained much interest from the scientific community and the livestock industry, due to its importance in obtaining healthy animal products. The gastrointestinal tract (GIT) is considered an organ that digests and absorbs nutrients, harbors large amounts of immune cells and microbes that are involved in immune function and metabolism of the host (Belanche et al., 2021). The GIT contributes to animal health, not only in food processing and subsequent nutrient uptake, but also by regulating epithelial and immune functions that are of importance for gut and general health through the communication between gut and gut microbiota (Vrieze et al., 2010).

The overarching definition of gut health includes effective digestion and absorption of food constituents, absence of diseases and pathogens in gastrointestinal tract, effective immune status, and stable and functional intestinal microbiota (Bischoff, 2011). The relevance of gut health is underlined by the fact that several symptoms associated with functional dyspepsia and irritable bowel syndrome such as bloating, regurgitation, constipation, diarrhea and abdominal pain reflect diseases that might affect quality of life (Bischoff, 2011). Therefore, a healthy gut contributes to healthy animals with optimal and efficient performance.

2.1.1 Health of epithelia and immune status

Functions of the GIT include absorption, fermentation and digestion of nutrients, barrier function against exogenous pathogens and host-microbial interactions. The GIT is also an immunologically organ that is constantly exposed to the endogenous and exogenous stimuli (Khafipour et al., 2016). Gut health can be influenced by many factors, including the retention time of digesta, nutrient digestion mechanisms, morphology and immune status of the GIT (Plaizier et al., 2018). In order to maintain homeostasis of gut microbial biomasses, the GIT contains mucosal layers to prevent the translocation of the pathogens and toxins into blood circulation. In ruminants, in contrast to the intestines, rumen has a multilayer structure of the epithelium with tight junctions to achieve this function and rumen papillae can greatly increase the absorptive surface for organic acids produced during rumen fermentation (Aschenbach et al., 2009; Penner et al., 2009). Although the hindgut does not have a multilayer epithelium, the mucus is the first line to defend micro-associated molecular pattern, the inner layer of its mucus attaches epithelial cells firmly and it is resistant to external bacterial colonization (Khiaosa-Ard and Zebeli, 2018). It has been reported that the monolayer intestinal epithelium is more sensitive to micro-associated molecular patterns and acidity that results in increased permeability than rumen epithelium (Gressley et al., 2011).

The reticulo-rumen is exposed to the pathogenic bacteria and toxins from the external environment, so a strong barrier function is necessary to prevent the translocation of these pathogens and toxins into the systemic circulation (Enemark, 2008; Plaizier et al., 2018). Volatile fatty acids (VFA) are absorbed through rumen epithelium and supply energy and precursors for glucose and fatty acids to animals. Also, this absorption helps to stabilize

the rumen pH as VFAs are absorbed in exchange for the secretion of bicarbonate (Penner et al., 2009; Aschenbach et al., 2011). Higher grain content and lower fiber content in diet may reduce VFA absorption in the rumen and reduce saliva secretion to buffer rumen pH, as fiber is needed to stimulate the saliva production and rumen buffering (Plaizier et al., 2008; Zebeli and Metzler-Zebeli, 2012; Lean et al., 2014). It has also been reported that rapid grain diet feeding increases the permeability of the rumen epithelium, thus, these changes cause a leaky epithelium (Górka et al., 2017). As a result, toxins can be translocated into the systemic circulation. It has been reported that ruminal acidosis causes the barrier function damage of the total GIT, although the permeability of the epithelium is influenced differently in different regions (Plaizier et al., 2018). There are many factors that can compromise the permeability of the GIT epithelium. Zhang et al. (2013) proved that a low feed intake also disrupts the barrier function of the GIT with a dose-dependence of feed restriction of the *ad libitum* feed intake (Zhang et al., 2013), and Wood et al. (2015) showed an age-dependent reduction in the permeability of the epithelium of the whole GIT.

In the rumen, the stratum corneum of the squamous ruminal epithelium and papillae is in direct contact with rumen microbiota. Normally, rumen microbiota colonizes the surface of this stratum corneum, but do not penetrate this layer to reach the stratum granulosum (Lyte et al., 2018). There are no endocrine or resident immune cells in the glandular ruminal epithelium. However, the intestinal mucosa contains the largest number of endocrine cells in the body. A large amount of resident immune cells provides innate and adaptive immune defense against pathogens as well as an intramural nervous system, modulated by the central and autonomic nervous systems of the host. Moreover,

goblet and crypt cells in the epithelial mucosa can secrete mucin and water to form inner and outer layers of mucus, thus, preventing the colonization of the pathogens on the mucosal surface (Lyte et al., 2018). Therefore, the epithelium is very important as a barrier to prevent the translocation of the toxic compounds and the site for the interaction between the microbes and the host, and more studies are needed for further observations.

2.1.2 Health of digesta

During rumen fermentation, production of organic acids may increase the acidity inside rumen. Sufficient dietary physically effective NDF (peNDF) is needed in rumen buffering as it promotes the chewing and saliva production, and prevent small particles escaping from rumen fermentation (Plaizier et al., 2008; Zebeli et al., 2012; Lean et al., 2014). This escape can affect nutrient degradation, increasing hindgut fermentation and cause hindgut acidosis. Rumen pH is considered as one of the most important indicators of acidosis (Gozho et al., 2005; Petri et al., 2013; Plaizier et al., 2018). However, rumen pH varies among locations inside rumen. Previous studies found that free rumen fluid of the ventral sac has a lower VFA concentrations and a higher pH compared with particle associated rumen fluid in the dorsal sac of the rumen (Tafaj et al., 2005). Duffield et al. (2004) also showed that the rumen pH in the ventral sac of rumen liquid digesta is lower than in the cranial ventral, caudal ventral, and cranial dorsa of the rumen (Duffield et al., 2004). Besides, dietary non-fiber carbohydrates and starch can also influence nutrient absorption and utilization as well as gut health. It was reported by different studies that replacing dietary starch by sugars can increase chewing behavior, nutrient utilization and rumen pH, and this may be due to its promotion of butyrate production and energy supply to the rumen epithelium (Mojtahedi and Mesgaran, 2011; Hall and Weimer, 2016).

Therefore, optimizing starch and sugar ratio in diet could reduce the risk of acidosis. Furthermore, other factors such as particle size, feed quality and processing techniques and rumen degradability can also affect the nutrient utilization, risk of the acidosis and gut health (Plaizier et al., 2018).

Another biomarker of gut health is bowel habit, especially colonic function, such as the frequency of defecation, feces consistency, feces form and weight (Cummings et al., 2004). The frequency of the defecation varies among ages, and is greater in early life in the neonatal period compared with older or adult periods (Weaver, 1988). Feces consistency is not uniform even within a single motion, and it related to water content, which is normally 70-80% in humans (Cummings et al., 2004). So both of the constipation and diarrhea indicate abnormal condition of the gut. Bowel habit is determined by dietary structures such as non-starch polysaccharide and the transit time of the substances passing through the GIT (Cummings et al., 2004). Excessive amounts of carbohydrate in diet that are incompletely fermented in foregut and not digested in the small intestine can reach hindgut and affect bowel habit, while dietary fiber, maintains bowel habit as it can speed up transit to prevent gut disorders such as colon cancer (Burkitt et al., 1972). Transit time is the average time that substances spend passing the digestive tract, which is the important determinant of faecal weight and bowel habit (Connell and Smith, 1974). It has been reported that a slow digesta transit time is associated with a high prevalence of hindgut disorders such as diverticular disease and colon cancer while fast transit has been associated with low prevalence of these disorders (Burkitt et al., 1972). Transit time is also associated with gut, and especially, colon metabolism, such as production of bacterial metabolites. It has been found that increased

faecal ammonia is related with lower transit time (Cummings et al., 1978). Besides, other parameters such as the presence of blood or mucus in feces, incontinence and rectal emptying also indicate disorders of the gut (Cummings et al., 2004).

2.1.3. Gut microbiota in ruminants

Around 4 billion ruminants exist today that are important in sustainable agricultural development and provide milk and meat for human beings. Their gut microbiome is usually defined as the total genome of the complex microbial community that resides in the GIT of ruminant and that plays a crucial role in immunological, metabolic and physiological processes in the host. This microbioma is central to ruminant production and health (McCann et al., 2017; O'Hara et al., 2020). The gut microbiome contributes to the intestinal mucosal integrity, intestinal homeostasis and immune response. The gut microbiota community consists of symbiotic bacteria, protozoa, archaea, fungi and phages that can produce a wide array of enzymes to breakdown plant lignocellulosic and nonstructural carbohydrates, nitrogenous compounds and lipids. These are converted into absorbable compounds such as proteins and volatile fatty acids (VFA). These can then be utilized by the host (Nicholson et al., 2012; Malmuthuge and Guan, 2016). Operational taxonomic units (OTUs) are clusters of sequence reads with a given similarity that are assigned to a taxonomical level and are usually used to describe the microbiome. Several parameters like richness, evenness, abundance and diversity are commonly used in microbial studies to compare the microbial community among different niches (Khafipour et al., 2016; Plaizier et al., 2021). Previous studies showed that high microbiota diversity, richness and evenness help to stabilize and increase the robustness

of the microbial community, especially when animals are suffering from nutrition challenges (Khafipour et al., 2011; Khafipour et al., 2016; Tun et al., 2020).

2.1.3.1 Reticular-rumen microbiota

There are four compartments of stomach in ruminants, including the rumen, reticulum, omasum and abomasum. The rumen and reticulum are usually considered as reticulo-rumen, and undeveloped at birth while sucked milk reaches to omasum and abomasum through reticular groove (McDonald, 2002). As soon as animals start to eat solid food, the reticulo-rumen enlarges greatly. In the adult, reticular-rumen comprises 85% of the total capacity of the stomach. Fiber consumption stimulates the development of reticulo-rumen, and the VFAs, especially butyric acid produced by rumen microbes, encourages the growth of rumen papillae (McDonald, 2002).

In ruminants, rumen fermentation contributes most in microbial fermentation in GIT, and is crucial to the growth and production of animals. The rumen is the most diverse and complex microbial ecosystem in ruminant's GIT, and includes 95% symbiotic bacteria (Zhou et al., 2015; O'Hara et al., 2020). Rumen microbiota plays an important role in the metabolism in ruminants by producing 70% of daily energy requirement of host. The rumen provides anaerobic conditions for microorganisms to promote their growth and also for the activity of enzymes such as cellulytic, proteolytic, amylolytic and lipolytic enzymes that are produced by microbiota (Zhou et al., 2015; Nagaraja, 2016; Moraïs and Mizrahi, 2019).

2.1.3.1.1 Reticulo-rumen bacteria

The reticulo-rumen harbors a wide range of microbes, including bacteria, archaea, protozoa and fungi (Figure 2-1). The composition of the rumen microbiota can be influenced by several factors such as diet, host ages and genotypes (Russell and Rychlik, 2001; Weimer, 2015; Zhou et al., 2015; Sarah Moraïs and Itzhak Mizrahi., 2019). The stability of the reticulo-rumen ecosystems relies on the ecological properties of its resident microbiota (Khafipour et al., 2016; Banerjee et al., 2018). Rumen bacteria are the predominant microbes in rumen that can reach to 10^{11} cells/gram density in rumen content, and the community is comprised with more than 500 species (McDonald, 2002; McSweeney et al., 2005). Rumen bacteria can be divided into solid digesta-associated populations that attaches to feed particles, liquid-digesta associated population that contains planktonic bacteria in rumen liquid digesta (McAllister et al., 1994), and epimural and mucosal-associated population that attach to rumen epithelium (Zhou et al., 2015) (Figure 2-1). Around 75% of total rumen bacteria are solid-associated population (Koike et al., 2003), and only 1% is epimural and mucosal-associated, which is considered as the most diverse population (Malmuthuge et al., 2012) and relates more to the host metabolic activity. The disruption in homogeneity of rumen microbiota may cause various metabolic disorders such as ruminal acidosis, diarrhea and systemic inflammation (Figure 2-1).

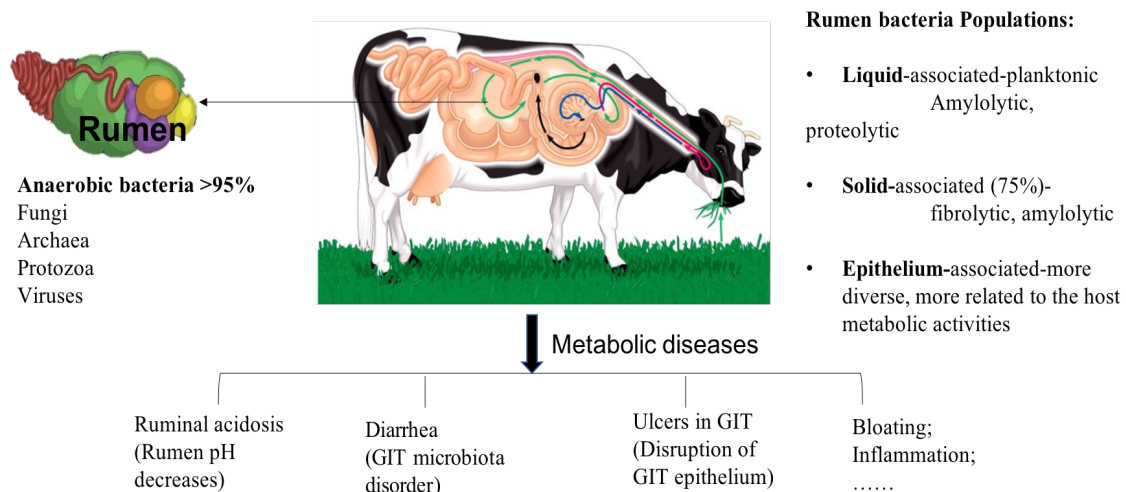


Figure 2-1. Ruminal microbiota and metabolic diseases in ruminants. Rumen bacteria comprises more than 95% of population in the microbial community, includes liquid-, solid-, and epimural bacteria. The interruption in homogeneity of rumen microbiota causes various metabolic diseases such as ruminal acidosis, diarrhea and systemic inflammation.

2.1.3.1.2 Composition and function of rumen bacteria

The microbial community in reticulo-rumen is complex and diverse according to cultivation-based and molecular techniques (Amann et al., 1995; McGovern et al., 2018). Most rumen bacteria are either gram-negative or gram-positive and they are not present as single colonies (Zhou et al., 2015). Studying the entire structure of the rumen bacteria would be able to help with understanding all of the microbial functional potentials. According to early studies, the composition of the microbial community can be different depending on experimental methods (Weimer, 2015; Brede et al., 2020) and host phenotypes. Despite the limitations of the early molecular identification on examining the composition of the rumen microbiota, Jami and Mizrahi (2012) used a high-throughput sequencing method to achieve deeper description of the complex ruminal microbial

community. Kim et al. (2011) found that *Firmicutes* and *Bacteroidetes* are the most abundant phyla in the rumen, followed by *Proteobacteria* (Kim et al., 2011). At the lower taxonomy levels, *Clostridia* and *Bacteroidia* are the most predominant classes, *Bacteroidales* and *Clostridiales* are the most predominant orders, *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* are the most predominate families (Kim et al., 2011; Creevey et al., 2014). However, taxonomy of ruminal microbiota continues to be studied, and the possibility of uncultured ruminal microbiota still exists.

The taxonomic composition of rumen microbiota is different among host phenotypes, so the presence of ‘core-microbiome’ helps to stabilize the functionality and the capability to carry out the metabolic activities of the rumen ecosystem (Khafipour et al., 2009c; Banerjee et al., 2018). The resilience of the microbiota in rumen ecosystem against the exogenous (e.g. microbes) and nutritional perturbations is also very important. Resilience represents the amount of perturbations/stress that a system can tolerate before it changes from one stable equilibrium condition to another (Folke et al., 2004; Plaizier et al., 2018). The chance of the exogenous microbes to colonize a rumen niche is limited, as they need to compete with the resident members that are already adapted to the rumen environment. Even if those exogenous microbes integrate into the gut microbiomes and affect the functionality of the microbial community, these changes usually will not last long (Derrien and van Hylckama Vlieg, 2015). Because of the complex composition and functional redundancy, rumen microbiome is robust against those perturbations (Weimer, 2015; Plaizier et al., 2018).

The rumen microbiota is essential for ruminants’ nutrient utilization, including fibrous and non-fibrous materials (Russell and Rychlik, 2001; Firkins and Yu, 2015).

Thus, studies on rumen microbiota are mainly focused on their effect on meat and milk production. The Rumen harbors an immense species- and strain-level diversity with overlapping metabolic capabilities (Plaizier et al., 2018). Rumen microbiota can produce fiber-degrading enzymes that ruminants themselves cannot, and provide the host with protein, vitamins and short-chain organic acids such as acetic, propionic and butyric acids (Tao et al., 2022). Within that, rumen bacteria play a dominant role in ruminal fermentation (Russell and Rychlik, 2001). Around 90% of the amino acids come from the microbiota and reaches the small intestine, and short-chain organic acids provide the energy for host metabolism (Nocek and Russell, 1988). Based on the enzymes produced by the microbiota, they can be characterized as cellulolytics, hemicellulytics, amylolytics, proteolytics and lypolytics (Figure 2-1, Firkins and Yu, 2015). Cellulolytic bacteria in the rumen, such as three common species *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* are strictly anaerobic and the optimal pH range for their cell growth is only 6-7 (Russell and Rychlik, 2001). Predominate hemicellulose digesters in the rumen are *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Ruminococcus flavefaciens* and *Ruminococcus albus* (Dehority, 2003a). Starch is rich in grain-based diets and requires amylolytic species to break its glycosidic bonds. Amylolytic species mostly belong to *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*, and some strains belong to *Fibrobacter succinogenes* and *Clostridium*. Moreover, *Streptococcus bovis* can ferment starch rapidly and produce lactic acid as the end product, while *Succinimonas amylolytica* and most stains of *Selenomonas ruminantium* degrade starch by hydrolysis and produce lactic, acetic and propionic acids as end products (Dehority, 2003b; Zhou et al., 2015). Because some amylolytic bacteria can secrete polysaccharides and produce

foam, they may cause bloat that may affect feed intake and even cause death (Russell and Rychlik, 2001). Some bacteria such as *R. albus* and *B. fibrisolvens* are armed with bacteriocins that may affect rumen fermentation (Russell and Rychlik, 2001). Furthermore, only certain species such as *Prevotella* can digest amino acids for ATP formation and bacterial growth (Walker et al., 2005), and all of the vitamin B compounds and vitamin K can be synthesized by bacteria (Zhou et al., 2015).

Although epimural bacteria are only small populations, they interact with the rumen epithelium and play an important role in maintaining barrier function and immune response of the epithelium (Petri et al., 2020; Plaizier et al., 2022). It has been reported that epimural bacteria are taxonomically distinct from rumen digesta bacteria, and that their population has not yet been accurately determined (Zhou et al., 2015). Previous studies reported that epimural bacteria are involved in many functions, such as ammonia and methane oxidization, and hydrolysis of urea, indicating epimural bacteria are involved in rumen fermentation in a different way (Zhou et al., 2015; Petri et al., 2020).

2.1.3.1.3 Factors affecting rumen bacteria

There are many factors that affect the composition and functionality of the rumen microbiota, including host genetics, age, environmental exposures such as geographic location, diet, and seasons (Zhou et al., 2015; Furman et al., 2020). Rumen microbiota starts their colonization after birth and reach a stable condition in a couple of months or years according to the host species (Jami et al., 2013). Even within the same pasture, animals do not establish identical microbial composition in all individuals. It was reported that ruminal bacteria diversity is influenced by animal breed (Guan et al., 2008),

and animals with the same breed host a more related rumen microbiome than those with different breeds. Besides, it has also been found that the rumen microbial ecology varies among individuals within the same breed (Weimer et al., 2010).

Several studies have reported the effect of the dietary changes on the reticular-rumen microbial community, and the composition and diversity of the rumen bacteria changes when dietary structure changes (Khafipour et al., 2009c; Zhou et al., 2015; Khafipour et al., 2016; Plaizier et al., 2021). As mentioned above, each microbial species has certain nutrient preference so that the community will shift their structure so it can be more suitable to utilize the substrate in the diet. In ruminants, higher proportions of grain in the diet are usually applied to promote increased energy supplies and higher animal production. And under high-grain diet feeding conditions, the relative abundance of fibrolytic bacteria such as *Ruminococcus flavefaciens* decreased as the diet switched to grain from forage diet (Zhou et al., 2015). Besides, the diversity of the rumen bacteria community is higher with forage diet feeding compared to that with grain diet feeding, indicating a more robustness rumen microbial community with forage diet (Khafipour et al., 2009c; Khafipour et al., 2016). Furthermore, the use of the supplementation such as prebiotic and probiotic can also influence the rumen microbial community.

Another important factor that affect the rumen bacteria is the rumen environment they colonize. It may, because of the acidic conditions inside the rumen (Zhou et al., 2015; Khafipour et al., 2016). Bacterial species such as some too vague fibrolytic bacteria are sensitive to the low pH (e.g. pH < 5.8), and their population declines as rumen pH decreases when there is a high proportion of grain diet supplied to the animals (Nagaraja

and Titgemeyer, 2007). Therefore, buffering rumen pH is essential to stabilize the rumen bacterial community.

2.1.3.2 Hindgut microbiota

There is a large number of microbes in hindgut for fermentation as amounts of starches and other fermentable substrates that escape from rumen fermentation and absorption in the small intestine reach the large intestine when animals are fed with grain diet (Glock and DeGroot, 1998; O'Hara et al., 2020). A large amount of starches that exceed the rumen fermentation, leading to by-pass rumen fermentation. Amylolytic activity in small intestine in ruminants is limited so that most of the incompletely fermented substrates are fermented in hindgut and increase the risk of hindgut acidosis (Gressley et al., 2011; Plaizier et al., 2018). There are differences in the composition and abundances of microbiota between the rumen and hindgut according to their epithelial structure difference and the profile of dietary and mucosa-associated glycans. Most of the dietary glycans are fermented in reticulo-rumen and converted to VFAs, only small amount of them need to be fermented in hindgut (Koropatkin et al., 2012). Thus, changes in amount of dietary glycans have little influence on composition of hindgut microbiota. Some species such as *Bacteroides* spp. can degrade endogenous glycans (in mucous secretions or epithelial cells) and are more effective in colonizing than other species in hindgut mucosa and influence hindgut homeostasis. Similar to what is observed in the rumen, dietary changes can also affect the composition and functionality in hindgut microbiota (Neubauer et al., 2020; Plaizier et al., 2021). Compared with ruminal

microbiota, hindgut microbiota has a higher relative abundance of *Firmicutes* and a lower relative abundance of Bacteroidetes (Plaizier et al., 2017; Brede et al., 2020).

It has been reported that diseases in the hindgut can also affect the animal health status as rumen diseases. The ability of hindgut to regulate its pH is limited due to the absence of protozoa and salivary buffering. Thus, the threshold of hindgut acidosis (6.0-6.6) (Metzler-Zebeli et al., 2013) is higher than ruminal acidosis (5.6-5.8) (Plaizier et al., 2012). The monolayer epithelium of the large intestine mucosa makes the hindgut epithelium more susceptible to the barrier damage and toxin translocation than rumen (Li et al., 2012; Plaizier et al., 2018). Emmanuel et al. (2007) found that the translocation of endotoxin across colon epithelia was higher than that across rumen epithelia (Emmanuel et al., 2007a), which indicates that the concentration of endotoxins in the hindgut can be higher compared with that in the rumen. Hence, microbial dysbiosis in the hindgut can also lead to systemic translocation of endotoxins.

2.2.3 Alternative techniques in gut microbiome study

Microbial communities in GIT have been studied for many years, since they play an important role in nutrient digestion and it supplies of absorbable substrates and energy for biological processes of the host animals (Zhou et al., 2015; Gruninger et al., 2019). It is, therefore, meaningful to assess the composition and functions of the microbiota in the GIT in order to optimize gut health. There is a large population of microbes that colonize in GIT after birth, which are highly diverse in form and functionality that gives much challenges/difficulties in scientific researches (Zhou et al., 2015; O'Hara et al., 2020). Prior to molecular biology, microorganisms were studied with culture-based methods.

However, rumen environment is strictly anaerobic and the pH is normally at 5.5-5.6, which is very specific for the growth of microbes. Therefore, more than 70% of gut microbes cannot be cultured or identified (Khafipour, 2009; Zhou et al., 2015). To date, culture-independent molecular techniques such as next-generation sequencing (NGS), including restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and ribosomal RNA clone libraries are commonly used in microbiome studies. Compared with the culture-based techniques, these molecular techniques are more sensitive and accurate (Zhou et al., 2015; Li et al., 2018).

Molecular techniques in microbial studies mainly rely on the sequencing of PCR amplicons of targeted marker genes, such as 16S rRNA amplicon sequencing (Li et al., 2018). In recent years, 16S rRNA sequencing has been used to identify bacteria isolates, compare the composition and diversity parameters in large numbers of environmental samples. The 16S rRNA gene is expressed among all species of bacteria with similar sequence length and contains both variable and conserved regions with discriminatory potentials. The choice of variable region, primer pairs 515F/806R targets V4 region of 16S rRNA and Pro341F/Pro805R targets V3-V4 region, affects the accuracy in community diversity estimations (McGovern et al., 2018). The database for the 16S rRNA gene has been well established for accurate comparisons of obtained and existing sequences (Zhou et al., 2015; Li et al., 2018). Recently, Illumina MiSeq has been mostly used in NGS sequencing studies as it yields the highest number of reads at the lowest cost. However, Illumina MiSeq has limitation for genus level analyses, as its reliable high-quality reads are only at 250 bp for the forward read and 230 bp for the reverse read (McGovern et al., 2018). Not only the primers selection, but also the variety of DNA

extraction methods and PCR protocols can bias this approach. The classification of sequences should also be considered in 16S rRNA phylogenetic analysis (McGovern et al., 2018). Basic Local Alignment Search Tool (BLAST) has been used to identify species from DNA that were extracted from mock community cells, which were incorporated with classification of the OTUs for taxonomic assignment (Popova et al., 2017; McGovern et al., 2018). Ribosomal Database Project (RDP), NCBI, Greengenes and SILVA databases are commonly used for taxonomic classification in rumen microbial analysis (Li et al., 2018; McGovern et al., 2018).

In ruminants, 16S rRNA sequencing is widely used to examine the relationships between rumen microbiota and milk production, methane emission, feed efficiency and health status (Jami et al., 2014; Xue et al., 2019). Terminal restriction fragment length polymorphism (TRFLP) community fingerprinting was used in studies to detect populations of microbes in different locations of the rumen. Khafipour et al. (2009) found that the proportion of *Bacteroidetes* was greater in alfalfa pellet-based feeding than that in grain-based feeding, and *Prevotella albensis* was the predominate bacteria in alfalfa pellet diet feeding while *Streptococcus bovis* and *Escherichia coli* were predominated bacteria in grain diet feeding (Khafipour et al., 2009c). It was reported that metabolic disorders such as subacute ruminal acidosis reduces the diversity and richness in rumen and hindgut microbial communities (Khafipour et al., 2016; Plaizier et al., 2016).

Because biases exist in this approach, 16S rRNA amplicon sequencing is not suitable for monitoring the dynamics of the microbial communities. Another sequencing approach to evaluate the composition of the rumen microbiota is metagenomic shotgun sequencing.

Metagenomics is the analysis of microbial communities in particular habitat with high-throughput sequencing, and it has been widely used to reveal the diversity and metabolism of the microbial community in different niches (Wu et al., 2021). In this approach, taxonomy of the microbial community can be assigned through alignment to partial/fully assembled microbial genomes with MG-RAST and Kraken (Li et al., 2018; Zhou et al., 2021). Shotgun metagenomics was firstly applied to identify carbohydrate-active enzymes and genes encoding glycoside hydrolases. Later on, Hess et al. (2018) detected 27,755 carbohydrate-active genes in rumen microbiota and found that 57% of them were associated with cellulytic degradation (Li et al., 2018). Zhou et al. (2021) compared amplicon and shotgun sequencing for rumen microbiome study in Angus bulls and found that the shotgun sequencing method identified more bacteria genera (303 vs. 48) and archaeal species (171 vs. 4) than amplicon sequencing, while amplicon sequencing revealed the protozoal composition. Besides, when they detect the dietary effect on rumen microbiome, these two approaches found opposite trends of the abundance of *Bacteroidetes* and *Firmicutes* in bulls fed with background diet and finishing diet (Zhou et al., 2021). Wu et al. (2021) used metagenomics to identify the relationship between rumen microbiota and milk protein and fat synthesis and found that abundances of *Prevotella* species are positively related to total VFA concentrations (Wu et al., 2021). Steward et al. (2019) assembled 4,941 rumen microbial metagenome-assembled genomes to predict and annotate a large set of rumen proteins, aiding in understanding the structure and functions of the rumen microbes (Steward et al., 2019).

To predict the rumen microbial functions, metatranscriptomics is also needed to reveal overall metabolic functions of rumen microbiome, as they can capture the whole

transcriptomic information for microorganisms (Li et al., 2018). Metagenomics and metatranscriptomics have been used in many studies to reveal carbohydrate-degrading enzymes in ruminants and they were demonstrated to increase the resolution of microbial functional profiling (Li et al., 2018; Xue et al., 2020). Furthermore, metabolomic approaches were applied to detect the relative concentrations of rumen microbial metabolites, such as amino acids, carbohydrates and fatty acids, and the taxa (*Prevotella* species) correlated with which positively (Xue et al., 2020). Overall, using multi-omics can help to better understand the communication between microbes and microbes and between microbes and the host, thereby, helping to develop the strategies to improve animal production.

2.3 Role of diet in modulation of gut microbiota and gut health

2.3.1 High-grain diet feeding and gut health

Ruminant animals are commonly fed high proportions of grain in their diet to promote their production. However, high grain feeding increases the starch content of digesta and the fermentation in the GIT, and as a consequence, increases the production and accumulation of the organic acids such as VFAs in the forestomach (Plaizier et al., 2008; Plaizier et al., 2012). Besides, animals chewing behavior is less when they are fed with grain diet than with forage diet so that the saliva production is lower in animals fed with grain diet. Saliva is important to buffer the rumen pH. Therefore, increased acids accumulation and reduced saliva production decrease the rumen pH and increase the osmolality of the rumen digesta (Plaizier et al., 2012).

2.3.1.1 Subacute ruminal acidosis

Subacute ruminal acidosis (SARA) is commonly occurring in high-yielding dairy cows to meet their high-energy requirement and can affect their production and health (Plaizier et al., 2008; Zebeli et al., 2012; Plaizier et al., 2018). Excessive feeding of highly fermentable carbohydrates will lead to an increase in rumen fermentation accumulation of fermentation acids and acidity, as well as promote the growth of lactic acid-producing and lactic acid-utilizing bacteria but inhibit the growth of fibrolytic bacteria in the rumen. This results in the accumulation of acids and a decrease in rumen pH. This disorder mainly occurs due to the accumulation of volatile fatty acids (VFAs, including acetate, butyrate, propionate), while acute ruminal acidosis mainly occurs due to the accumulation of lactic acid (Valente et al., 2017). At present, the depression of rumen pH is mostly used to diagnose SARA. A ruminal pH between 6.0 and 7.2 is considered the normal range, while a pH below 5.5 or 5.6 for prolonged periods of time is considered ruminal acidosis (Cooper et al., 1997; Nocek, 1997; Valente et al., 2017). During acute ruminal acidosis, rumen pH is below 5 due to lactic acid accumulation. The decrease of ruminal pH and symptoms of SARA are moderate compared with that of acute ruminal acidosis (Plaizier et al., 2022). The rumen pH thresholds of SARA are based on many factors such as the health of rumen epithelium, health of rumen microbial community and pH measurements technologies (Khafipour, 2009). However, the threshold of the ruminal pH (below 5.6 or 5.8) for SARA diagnosis varies among different studies and due to different methodologies used in each lab (Krause and Oetzel, 2006; Plaizier et al., 2008; Plaizier et al., 2012). In research settings, SARA have mainly been induced by excessive grain diet feeding (Kleen et al., 2003; Khafipour et al., 2009c;

Plaizier et al., 2021). The source of grain matters in the induction of SARA. It has been reported that starch from wheat and barley are more degradable than corn starch, and that ground grains are more rapidly fermented in the rumen than rolled grains (Plaizier et al., 2022). Khafipour et al. (2009) reported that symptoms of SARA induced by high grain feeding were much severe than that induced by feeding pellets of ground forage (Khafipour et al., 2009b; a). Symptoms of SARA could be affected by other gut disorders, so the diagnose of SARA maybe inaccurate if we use only one of them such as pH (Plaizier et al., 2012; Plaizier et al., 2022). A combination of several symptoms including inflammation, poor fecal consistency, milk fat depression and reduced milk production for the diagnosis of SARA is more reliable (Plaizier et al., 2018; Plaizier et al., 2022). The detection methods, sample size and different sites of the rumen cause variation of the ruminal pH, therefore using the duration and areas below rumen pH thresholds (<5.6 or 5.8 and >3 h/day) is more indicative of the severity of SARA (Gozho et al., 2007; Zebeli et al., 2008).

SARA can cause economical loss in dairy farms, and it has, therefore, been studied for many years. The prevalence of SARA has been reported in many studies and it was demonstrated that the prevalence of SARA differs among lactation progresses (Plaizier et al., 2018). Based on the rumen pH criteria of SARA, cows in early lactation when they are switched to high grain diet feeding and in mid-lactation when the feed intake increases, are usually at higher risk of SARA (Krause and Oetzel, 2006). In early lactation, the high VFA production due to high-grain diets feeding exceeds the absorptive capacity for VFA of the rumen epithelium. In mid-lactation, VFA accumulates in the rumen because of the VFA production increases as a result of increased feed intake

(Khafipour, 2009). Feeding a forage-based diet with coarse particles increases ruminating activity, chewing behavior and saliva production, which increase rumen buffering (Beauchemin et al., 2003), and thus, reduces the risk of SARA.

Previous studies showed that low rumen pH during SARA is associated with reductions in feed intake, depression of milk fat content and milk yield, and systemic inflammation (Krause and Oetzel, 2006; Plaizier et al., 2012; Guo et al., 2017). The milk fat depression may not be caused directly by the low rumen pH. That is because rumen pH depression causes the incomplete biohydrogenation of polyunsaturated fatty acids and increases in trans-octadecenoic acids, which inhibit *de novo* milk fat synthesis in the mammary gland (Zebeli et al., 2006; Iqbal et al., 2009; Zhou et al., 2014). However, not all of the studies agreed with these findings as some of them found no effect of SARA on milk production. These differences may be due to the low sample size and milking recording methods (Plaizier et al., 2018). Therefore, milk fat depression cannot be the sole measurement but can be used together with other parameters such as rumen pH, urine and fecal pH, and acute phase proteins for the diagnoses of SARA.

2.3.1.2 SARA and immune responses

SARA has been associated with inflammation. This inflammation can be localized in the rumen epithelium (rumenitis), or be systemic (Emmanuel et al., 2007b; Eckel and Ametaj, 2016; Guo et al., 2017). This systemic inflammation could be due to the translocation of immunogenic compounds, like lipopolysaccharides (LPS) and bioamines from GIT (Figure 2-2, Lüderitz et al., 1984; Alexander and Rietschel, 2001; Plaizier et al., 2012., Plaizier et al., 2018). Several studies have found that grain-induced SARA

increased LPS concentrations in rumen digesta (Gozho et al., 2007; Khafipour et al., 2009b; Li et al., 2016; Guo et al., 2022). The increased LPS concentration in the rumen is thought to be caused by rapid growth or lysis of gram-negative bacteria (Mashubrn-Warren et al., 2008). When SARA persists, several species of bacteria, including fibrolytic bacteria, cannot survive, and free LPS can be rapidly released (Zebeli et al., 2011; Plaizier et al., 2012).

As mentioned above, the rumen epithelium contains a stratified structure, including a keratinized layer, which is in direct contact with the ruminal environment. High-grain diets increase the proliferative activity of epithelial cells and prematurely transition these cells to the stratum corneum, resulting in parakeratosis (Kleen et al., 2003; Steele et al., 2011). Studies have found that SARA causes histological changes in the rumen epithelium, leading to increased permeability of the rumen epithelial cells and lesions in the epithelium impairing the barrier function of the rumen epithelium (Steele et al., 2011; Plaizier et al., 2012). There is strong evidence that SARA disrupts the integrity of the rumen epithelium and allows the translocation of pathogenic compounds such as LPS and bioamines, increasing the risk of inflammation. Studies have found that the translations of LPS from the digestive tract into the blood circulatory system caused by SARA can lead to systemic inflammation (Plaizier et al., 2008; Plaizier et al., 2012; Guo et al., 2017). LPS is a component of the outer cell wall of Gram-negative bacteria, allowing only small molecular weight, hydrophilic molecules to pass through the outer membrane (Plaizier et al., 2008). Although LPS is bound to the bacterial outer membrane, it is shed during the

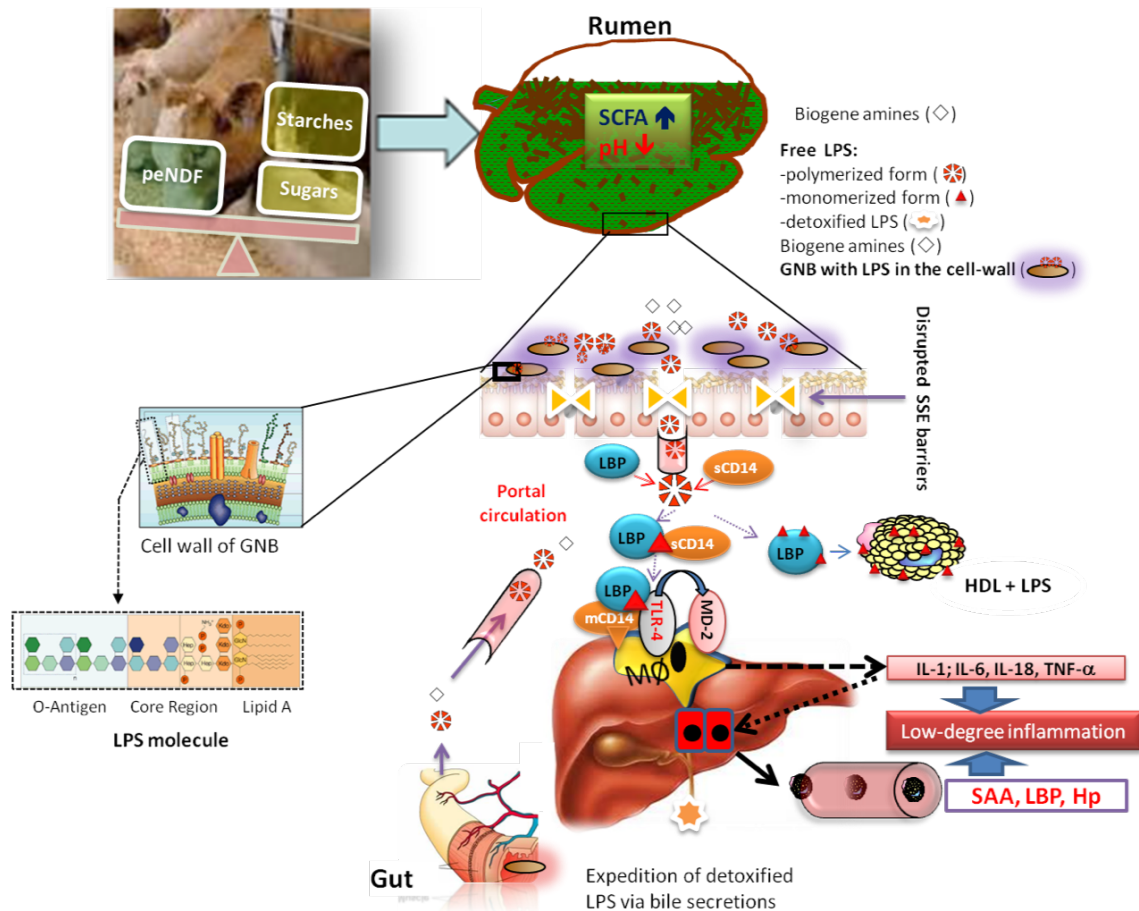


Figure 2-2. Proposed mechanisms through which high grain feeding causes inflammation in dairy cows, and the role of lipopolysaccharide (LPS) endotoxin in these mechanisms

(Plaizier et al., 2018). GNB =gram-negative bacteria; HDL =high-density lipoprotein; Hp=haptoglobin; IL=interleukin; LBP=LPS binding protein; mCD14=membrane CD14; MD-2 =Lymphocyte antigen 96; peNDF =physically effective NDF; SAA=serum amyloid A; sCD14 =soluble CD 14; SCFA=short-chain fatty acids; SSE=simple sequence editor; TLR =Toll-like receptor; IL =interleukin; TNF α =tumour necrosis factor α .

bacterial growth and stationary phases and it is released following cell division and cytolysis (Eckel and Ametaj, 2016). LPS consists of O-specific chain, core region and

lipid-A, in which lipid-A is the toxicity principle of LPS (Figure 3, Lüderitz et al., 1984; Seydel et al., 2000; Eckel and Ametaj, 2016). Once cytolysis occurs, lipid-A is exposed so that LPS in the free state is toxic. When entering the peripheral circulatory system, it causes a systemic immune response (Alexander and Rietschel, 2001).

Ruminal LPS concentrations varies greatly among different SARA studies, which may due to differences in the techniques used for measuring LPS, processing of samples and SARA induction methods (Plaizier et al., 2018). It has been reported that increases of free ruminal LPS is higher in grain-induced SARA compared with in ground forage-induced SARA (Khafipour et al., 2009c). Some studies found that grain-induced SARA can also increase the concentration of LPS in feces, indicating that hindgut acidosis may be caused by increases in starch that bypasses rumen fermentation and digestion in the small intestine (Gressley et al., 2011; Plaizier et al., 2018). Because of the monolayer structure of the large intestine epithelium, the barrier function of the large intestinal epithelium is easier compromised than that of rumen (Li et al., 2012; Tao et al., 2014). Therefore, LPS in the hindgut is more likely to cause inflammation than ruminal LPS (Plaizier et al., 2018).

LPS is degraded by liver cells and may only be detectable in the peripheral blood circulation once the LPS clearing capacity of the liver has been exceeded (Eckel and Ametaj, 2016; Guo et al., 2017). Hence, the absence of LPS in peripheral blood does not mean that no translocation of LPS occurred. Along with the breakdown of the epithelial barrier of the GIT, LPS can be transported to the internal environment of the body such as blood circulation or lymphatic circulation (Eckel and Ametaj, 2016). Once translocated, LPS will react with receptors from a variety of cells such as monocytes, endothelial cells,

smooth muscle cells and dendritic cells to promote the production of pro-inflammatory cytokines. To achieve this process, LPS binds to LPS-binding protein (LBP), which can increase the activity of LPS and even exhibit the biological activity of very few LPS (Tomlinson and Blikslager, 2004). LBP can promote the binding of LPS to lipoproteins and enhance the neutralization of LPS. In addition, the LPS-LBP complex can be transported to the cell membrane surface with the help of CD14 molecules, and then react with the corresponding Toll-like receptors 4 (TLR4) to activate various mitogen-activated protein kinases and nuclear factor κ B (NF- κ B) (Figure 3, Chang et al., 2015; Eckel and Ametaj, 2016). The ensuing cascade drives the gene transcription of a range of pro-inflammatory cytokines and chemokines, such as tumor necrosis factor- α (TNF- α), IL-1, and IL-6 (Figure 2-2, Tomlinson and Blikslager, 2004; Eckel and Ametaj, 2016).

The production of pro-inflammatory cytokines can trigger the acute phase responses in the body, including the production of acute phase proteins such as serum amyloid A (SAA), haptoglobin (Hp) and LBP in hepatocytes (Figure 3, Alsemgeest et al., 1994; Tomlinson and Blikslager, 2004). Nearly 30 acute phase proteins have been found, but SAA and Hp have the most obvious responses in cattle (Alsemgeest et al., 1994; Ganheim et al., 2007; Vels et al., 2009; Ceciliani et al., 2012). Studies have found that both grain-induced SARA and overfeeding of grains can cause the increase of acute phase proteins in the peripheral blood of cattle (Gozho et al., 2006; Khafipour et al., 2009b; Li et al., 2016). Acute-phase protein levels may be elevated even if SARA does not cause translocation of LPS from GIT into blood circulation (Plaizier et al., 2018). However, regardless of how similar the rumen pH drop was, grain-induced SARA induced increases in peripheral blood acute-phase proteins, whereas replacement of

alfalfa hay with alfalfa pellets did not induce increases in peripheral blood acute-phase proteins (Khafipour et al., 2009c; Li et al., 2016). The reason may be that SARA induced by alfalfa pellets did not change the concentrate-to-forage ratio and the starch content of the diet so that it did not affect the rumen fermentation. Besides, administration of *Escherichia coli* LPS causes at least ten times higher increases in SAA and Hp and some severe clinical symptoms such as fever than those reported in grain-induced SARA (Gozho et al., 2007; Plaizier et al., 2018). *E.coli* LPS are considered more toxic than LPS from other bacteria. It has been reported that repeated exposure to LPS can cause endotoxin tolerance, and once this tolerance occurred, immune responses to LPS of the animals may be limited or absent (Andersen, 2003). When grain-induced SARA occurs, the body's inflammatory response may not only be due to the transfer of LPS, but the weakening of the barrier function caused by other immunogenic components such as bioamines and histamine (Figure 2-2, Kleen et al., 2003). According to previous studies, a high-grain diet increases the concentrations of many immunogenic compounds besides endotoxins such as methylamines, histamine and tyramine in the rumen and peripheral blood, and cause perturbations in concentrations of some amino acids such as lysine, valine and leucine (Ametaj et al., 2010; Saleem et al., 2012). That means these compounds are translocated into blood circulation from GIT by compromised barrier function of gastrointestinal epithelium, and, as a consequence, cause health problems.

2.3.1.3 Effect of SARA on gut microbiome

In ruminants, most metabolic disorders, such as acute and subacute ruminal acidosis occur in early or mid-lactation, that are associated with changes in rumen microbial

composition and functionality (Khafipour et al., 2009c; Plaizier et al., 2016). The dysbiosis of GIT affects microbial metabolites (Plaizier et al., 2008; Saleem et al., 2012), and these compounds affect metabolism and immune function in or outside the gut through the enterohepatic circulation or the brain-gut axis (Lyte, 2014). Neuroactive substances produced by gut microbes have been shown to influence hormonal responses in the brain through the gut-brain axis, leading to changes in hormone levels (Lyte, 2014; Lyte et al., 2016). This can affect not only the diversity and behavior of microbes in the digestive tract, but also the microbiome in other parts of the body, such as the vaginal and breast systems, leading to the occurrence or development of infectious diseases, such as mastitis (Collins and Bercik, 2009; Lyte, 2014). Therefore, gut microbes can be considered the largest endocrine organ in the body.

At present, with the culture-based and high-throughput sequencing methods we can determine the functional properties of the microbiome, and also detect changes in microbial composition that lead to significant changes in the functionality of the population (Khafipour et al., 2009c; Mao et al., 2013). The composition and functionality of the rumen microbial community can be affected by many factors such as pH and feed types (Russell and Rychlik, 2001; Loor et al., 2016). Many studies have shown that the richness, uniformity, and diversity of foregut and hindgut microbiota are reduced in cattle fed high-grain diets, thereby reducing the functionality of these microorganisms (Russell and Rychlik, 2001; Mao et al., 2013; Loor et al., 2016). However, the magnitude of this reduction effect varies among experimental studies and individual animals (Russell and Rychlik, 2001; Loor et al., 2016). That said, conflicting and inconsistent results in some studies may be caused by other factors, such as differences in diets, host, experiment

methods, including those used to define microbial properties. The later includes DNA extraction methods, standardization of the amount of sequencing primers, and choice of primers (generic bacterial primers versus target-specific primers; or different choices for the hypervariable regions of the 16S rRNA gene (Khafipour et al., 2011; Loor et al., 2016).

Ruminal dysbiosis is considered as the interruption of diversity and functionality of the rumen microbiome, and as a consequence, affect the absorption and utilization of nutrients as well as the health status of the animals (Firkins and Yu, 2015; Zhou et al., 2015). According to this definition and symptoms, SARA is considered to affect rumen microbiota and may cause ruminal dysbiosis, offering suitable environment for pathogenic microbes in GIT (Plaizier et al., 2016; Plaizier et al., 2022). It has been shown that SARA conducted by high grain feeding reduces the richness and diversity of the rumen microbiota and affects the functionality of the microbial community (Khafipour et al., 2009c; Khafipour et al., 2016; Xue et al., 2020). When high-grain diets are consumed, starch appearing in the rumen is fermented by amylolytic bacteria to produce pyruvate, and finally VFAs are produced to reduce the rumen pH (Nocek, 1997). This means that many Gram-negative bacteria are less susceptible to low pH, including lactic acid-utilizing bacteria such as *M. escherichii* and *Selenomonas ruminants* (which can convert lactic acid to pyruvate). Conversely, there were increased numbers of some gram-positive bacteria, notably *S. bovis*, also known as lactobacilli (Goad et al., 1998; Enemark et al., 2002). Under the conditions of a low rumen pH, lactic acid utilizing bacteria can utilize lactic acid as an energy substrate in addition to other substrates. This change in rumen bacterial populations further reduces rumen pH through an increase in L-lactic acid,

which is 10 times stronger than VFA (Iqbal et al., 2010; Marx et al., 2011). This results in the growth of only pH-tolerant bacteria such as *Lactobacillus*, an efficient producer of lactic acid, especially D-lactic acid (Marx et al., 2011). When the rumen pH drops to below 3.8, this lactic acid can enter the circulation through the rumen wall leading to metabolic acidosis (Hernández et al., 2014).

Diet composition is the main factor affecting rumen microbial composition, and in adult animals, the population of the microbes is relatively stable. In the rumen, grain-based SARA increases the relative abundance of *Firmicutes* and reduces that of *Bacteroidetes* in both rumen digesta and the epimural microbial community, because *Bacteroidetes* is more efficient in degrading fiber (Khafipour et al., 2009c; Mao et al., 2013; Plaizier et al., 2017; Petri et al., 2020). Epimural microbiota interacts with the host epithelium and contributes to the barrier function and immune response, thus, they play an important role in attenuating the negative effect of SARA (Plaizier et al., 2021; Plaizier et al., 2022). Fernando et al. (2010) found that grain-based SARA reduced the population of fibrolytic bacteria such as *Fibrobacter succinogens* and *Butyrivibrio*, while it increased that of amylolytic bacteria including *Prevotella bryantii*, *M. elsdenii*, *S. ruminantium* and *S. bovis* (Fernando et al., 2010). *Prevotella* is the most abundant genus of *Bacteroidetes*, and *Ruminococcus* is the most abundant genus of *Firmicutes*. Studies have shown that the starch content of diets is associated with the changes in relative abundance of various bacterial genera (Khafipour et al., 2009c; Mao et al., 2013). As long as severe acidosis does not occur, substantial increases in grains can affect the abundance of some, but not all, starch- and fiber-decomposing bacteria, according to the results of multiple studies, and this effect has been found to vary among studies (Khafipour et al.,

2009c; Metzler-Zebeli et al., 2013; Khafipour et al., 2016; Plaizier et al., 2017). These changes in the composition of microbiota are caused by changes in the grain and fiber contents of the diet, but do not indicate irreversible dysbiosis.

In ruminants, gut microbe species compete for nutrients and share metabolic functionalities, which is beneficial for enhancing the properties of GIT (Dehority, 2003a; Dehority, 2003b; Firkins and Yu, 2015). It can, therefore, be speculated that changes in microbial composition do not necessarily reflect changes in their functionality due to the complexity of bacterial functions. Predicted functionalities of microbiota used to be based on 16S rRNA sequencing, which accuracy is limited because of the limit accuracy of 16S rRNA sequencing when determine the composition of the microbial community at the species or strain level. Thus, it could be inaccurate to detect the effect of SARA on functionality of rumen microbiota (Zhou et al., 2015; Plaizier et al., 2022). Hence, to assess functional changes of the microbiota and the interaction between this change and ruminal dysbiosis or animal production during SARA, the application of a combination of metagenome, metatranscriptomic, metaproteomics and metabolomic approaches is needed to examine the metabolic changes at the species and strain level (Zhou et al., 2015; Khafipour et al., 2016; Malmuthuge and Guan, 2016).

2.3.2 The approaches for optimizing gut microbiota

Gut microbiota is essential for the efficient nutrient absorption and utilization. Healthy microbiota includes high richness, evenness, diversity and vitality of the microbial community. High-grain diet feeding is commonly used in dairy farms to meet the energy requirements of high yielding dairy cows, but these diets may disturb the

stability of the gut microbiota and health. Hence, a variety of manipulation strategies have been developed to attenuate adverse effect of SARA. Feed additives are usually applied for maintaining microbiota and gut health, such as buffers, antibiotics, probiotics, prebiotics and postbiotics. Probiotics, prebiotics and postbiotics will be discussed briefly below.

2.3.2.1 Probiotics and prebiotics

Probiotics are live microorganisms which, when administered in adequate amounts into animals, confer a benefit on the health of the host and have become popular in animal nutrition as alternatives to antibiotics (Michalak et al., 2021). Probiotic microbes are usually classified as lactic acid utilizing bacteria, lactic acid producing bacteria and yeast (Retta, 2016). Probiotics can produce a wide range of antibacterial and bacteriostatic substances, such as organic acids and bacteriocins, and are involved in the production of amino acids and vitamins, as well as digestive enzymes (Petri et al., 2020; Michalak et al., 2021). Studies have shown that probiotic supplementation can modify the microbial population, maintain rumen health and enhance animal production and performance (Michalak et al., 2021; Hadhoud et al., 2022). Yeast is mostly common used in ruminant production, which can compete for adhesion sites for nutrients with some microorganisms such as pathogens in GIT (Michalak et al., 2021). Live yeast acts as probiotic, as its effects on modulates the composition and functionality of the rumen microbiota and increases feed intake (McCann et al., 2017; Hadhoud et al., 2022). It has been proven to improve feed efficiency and attenuate rumen acidosis. Yeast cells are able to suppress the lactate-producing bacteria and promote the growth of lactate-utilizing

bacteria such as *Megasphaera elsdenii* during grain diet feeding in ruminants (Hadhoud et al., 2022). Jiang et al. (2017) reported that live yeast cells tend to increase the abundance of fibrolytic bacteria (Jiang et al., 2017). Malekkahi et al. (2016) found that supplementation with active dried yeast increased the proportion of *Megasphaera elsdenii* in cows during SARA (Malekkahi et al., 2016). *Saccharomyces cerevisiae* strain is widely used *in vitro* and *in vivo* in many studies for ruminant nutrition, which can stabilize the rumen pH and increase fiber digestibility.

According to recent studies, usage of multi-component probiotics showed better effect than single probiotic on calves. Nevertheless, prebiotics combined with probiotics are increasingly used (Plaizier et al., 2018; Michalak et al., 2021). Prebiotics refers to substrates that are selectively utilized by host microorganisms conferring a health benefit, which are always incorporated with probiotics. Prebiotics include a variety of compounds, such as peptides, proteins, inulin and lactulose that provide nutrients for probiotics. Oligosaccharides are the most common prebiotics used in calves and showed its effect on feed efficiency and animal health (Michalak et al., 2021). Direct-fed microbes (DFM) are also commonly used as cow supplements, which include *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bacillus*, *Clostridium*, some species of *Bifidobacterium*, *Propionibacterium*, *Megasphaera elsdenii* or *Prevotella bryantii* (Michalak et al., 2021). Lactic acid bacteria as direct-fed microbes (DFM) together with lactic acid utilizing bacteria have proven to increase milk production and animal performance as well as propionate production in dairy cattle (Plaizier et al., 2018; Michalak et al., 2021; Monteiro et al., 2022).

2.3.2.2 Postbiotics

As a newer classification, postbiotics are functional bioactive compounds resulting from fermentation by food-grade microorganisms, which may promote health. Postbiotics can include many different compounds such as SCFAs, proteins, metabolites, extracellular polysaccharides and vitamins (Wegh et al., 2019). Nowadays, postbiotics draw more attention because of their stability and wide range of functionalities, and the microbial metabolites that contribute to communication between gut microbes, and microbes and the host (Wegh et al., 2019). Postbiotics can directly affect host physiology and immune response as well as its microbial composition and functionality (Vailati-Riboni et al., 2021). The composition of gut microbiota varies according to host phenotype, so the effect of these feed additives on host health is different.

Saccharomyces cerevisiae fermentation products (SCFP) are dietary postbiotic compounds produced by fermentation of *Saccharomyces cerevisiae*. It has been reported that SCFP supplementation reduces ammonia concentrations and methane emissions without disrupting the relative abundance of methanogens (McCann et al., 2017). Mahmoud et al. (2020) reported that SCFP reduce clinical disease scores in pre-weaned dairy calves infected with bovine respiratory syncytial virus, reduced the secretion of pro-inflammatory cytokines and virus-specific IL-17 by T cells. The latter indicated its effects on both systemic and mucosal immune responses (Mahmoud et al., 2020). Guo et al. (2022) found that SCFP reduced concentrations of plasma lipoteichoic acid and SAA as well as blood IL-1 β in dairy cows during grain-based SARA challenges, indicating its effect on reducing pro-inflammatory status (Guo et al., 2022). Riboni et al. (2021) found that SCFP improves udder health and immune response in mid-lactation dairy cows

suffering from a *S. uberis* mastitis (Vailati-Riboni et al., 2021). It has been reported that SCFP supplementation attenuates the adverse effects of SARA in dairy cows, such as reducing the total dry matter and neutral detergent fiber digestibility, and a depressed rumen pH (Li et al., 2016; Khalouei et al., 2020). SCFP can also improve milk production and feed efficiency in mid-lactation cows, which may be attributed to the rumen stabilization of this postbiotic (Acharya et al., 2017).

It has also been suggested that SCFP stabilizes rumen fermentation by enhancing the growth of fiber-digesting bacteria and lactic acid-utilizing bacteria by providing yeast fermentation metabolites such as vitamins, amino acids and organic acids. Hristov et al. (2010) reported that SCFP can increase the synthesis of microbial proteins in the rumen. Their study found that fermentable grain feeds caused a decrease in milk fat, while SCFP supplementation attenuated this decrease (Hristov et al., 2010). These authors did not fully understand the mechanism by which this occurred. However, subsequent studies found an inverse relationship between the free LPS concentration in the rumen and milk fat content. In addition, studies have found that SARA can lead to the synthesis of acute phase proteins in the liver and the proliferation and growth of cells in the immune system, which redistributes the energy required for the growth of the body (Guo et al., 2017; Khiaosa-Ard and Zebeli, 2018). Therefore, it can be speculated that the alleviating effect of SCFP on milk fat reduction is associated with the reduction of LPS in the digestive tract and the reduction of acute phase proteins in peripheral blood. However, the effectiveness of these supplements varies among studies (Tun et al., 2020; Vailati-Riboni et al., 2021; Schlabit et al., 2022). This may be due to the complex mechanisms of

attenuating the impact of high-grain diet feeding and the resulting SARA, the mode of action of SCFP needs further evaluation.

2.4 Summary

A healthy gut is characterized by a healthy epithelium with strong barrier function, healthy digesta and stable microbial environment. High-grain diet feeding is commonly used in dairy farms to meet the high nutrient demands, especially energy, of milk production. However, excessive grain feeding will reduce the effective physical fiber content in the diet and increase rumen fermentation. As a consequence, excessive grain feeding causes the accumulation of VFAs in the rumen and reduces rumen pH. When the pH value in the rumen drops to a certain level (e.g. $\text{pH} < 5.8/5.6$), gastrointestinal disorders such as acidosis and systemic inflammation occur. Low rumen pH will cause the lysis of Gram-negative bacteria in the rumen, thereby promoting the release of free LPS. When LPS exceeds the clearance capacity of the liver, it causes inflammatory damage to the liver and stimulates the liver to secrete acute phase proteins (SAA, Hp, LBP), triggering a systemic acute phase response. Rumen and hindgut microbiota are involved in nutrient fermentation and daily energy supply to the host ruminant, as ruminants lack of enzymes to degrade the undigested substrates. Evaluating the composition and functionality of the microbial community gives insights on rumen and hindgut fermentation and animal health status. Approaches used for microbial study such as amplicon sequencing and multi-omics help to understand the communication between microbes and microbes and the host. However, the discovery from these microbial studies varies among studies due to bias that exists among different approaches. SARA is

accompanied by reduced milk production, systemic inflammation, disturbed microbial communities including the reduction in their diversity and richness, as well as their metabolic functionality and economic losses. Therefore, manipulation strategies of SARA including feed additives such as probiotics and postbiotics are needed to attenuate the adverse effect of SARA. Probiotics and prebiotics have been studied for years and the new classification postbiotics have received more attention recently according to its stability and the variety of compounds such as polyphenols and antioxidants. Although many studies reported beneficial effects of postbiotics on milk production, pH and microbial stabilization in dairy cows, their mode of action is not yet fully understood.

CHAPTER THREE

MANUSCRIPT 1

***Saccharomyces cerevisiae* fermentation products reduce bacterial endotoxin concentrations and inflammation during grain-based subacute ruminal acidosis in lactating dairy cows**

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3.1 Abstract

Subacute ruminal acidosis (SARA) is a metabolic disorder in dairy cows that is associated with dysbiosis of rumen and hindgut microbiomes, translocation of immunogenic compounds from the gut lumen into blood circulation, and systemic inflammatory response. In this study we hypothesized that *Saccharomyces cerevisiae* fermentation products (SCFP) attenuate the increases in ruminal and peripheral bacterial endotoxin concentrations and the inflammation resulting from repeated induction of SARA. Lactating Holstein dairy cows (parity 2 and 3+, n = 32) were fed diets with or without SCFP (all from Diamond V) and subjected to 2 episodes of SARA challenges. Cows received a basal total mixed ration (TMR) containing 34% neutral detergent fiber and 18.6% starch, dry matter (DM) basis. Treatments were randomly assigned to control (basal TMR and 140 g/d of ground corn with no SCFP) or 1 of 3 SCFP treatments: basal TMR and 14 g/d Original XPC (SCFPa), 19 g/d NutriTek (SCFPb-1X), or 38 g/d NutriTek (SCFPb-2X) mixed with 126, 121, or 102 g/d of ground corn, respectively. Treatments were implemented from 4 wk before until 12 wk after parturition. During wk

5 (SARA1) and wk 8 of lactation (SARA2), grain-based SARA challenges were conducted by gradually replacing 20% of DM of the basal TMR over 3 d with pellets containing 50% wheat and 50% barley. Ruminal fluid, fecal, and blood samples were collected weekly during Pre-SARA1 (wk 4, as baseline), Post-SARA1 (wk 7), and Post-SARA2 (wk 10 for blood and wk 12 for rumen and fecal parameters) stages, and twice a week during the challenges SARA1 and SARA2. Rumen papillae samples were taken only during Pre-SARA1 and Post-SARA2. We measured the concentrations of free lipopolysaccharides (LPS) in the rumen fluid and feces; free LPS and lipoteichoic acid (LTA) endotoxins in peripheral plasma; interleukin (IL)-1 β and IL-6 in peripheral serum; acute-phase proteins, serum amyloid A (SAA), and LPS-binding protein in peripheral plasma; haptoglobin (Hp) in peripheral serum; and myeloperoxidase (MPO) in rumen papillae. Induction of SARA episodes increased free LPS concentrations in rumen fluid and tended to increase LTA in peripheral plasma. The SARA episodes increased concentration of circulating SAA and tended to increase that of IL-1 β compared with Pre-SARA1. Induction of SARA did not affect the concentrations of circulating IL-6, Hp, and MPO. The SCFP supplementation reduced plasma concentrations of LTA and SAA and serum concentration of IL-1 β compared with control. Additionally, SCFPb-2X tended to reduce ruminal LPS in second-parity cows compared with control. Overall, SCFP supplementation appeared to stabilize the rumen environment and reduce proinflammatory status, hence attenuating adverse digestive and inflammatory responses associated with SARA episodes.

Key words: *Saccharomyces cerevisiae* fermentation products, SARA, endotoxins, inflammatory response, dairy cow

3.2 Introduction

A postbiotic is a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (Salminen et al., 2021). Examples of postbiotics used in dairy are *Saccharomyces cerevisiae* fermentation products (SCFP), which have been shown to benefit rumen fermentation (Zhu et al., 2017), milk production (Poppy et al., 2012), and efficiency of milk production of lactating dairy cows (Zhang et al., 2013). Supplementation with SCFP also have been shown to reduce inflammation during pathogenic (Vailati-Riboni et al., 2021), physiological (Li et al., 2016), and immunological challenges (Jiang et al., 2018). It is proposed that functional metabolites, organic acids, vitamins, and antioxidants present in postbiotics, such as SCFP, may either be used as nutrients by members of gut microbiota or act as signaling molecules affecting microbe-microbe or microbe-host interactions and the physiological and immunological responses of the host (Salminen et al., 2021).

Beneficial health effects of SCFP supplementation during SARA have been suggested (Li et al., 2016). This common metabolic disorder causes substantial economic loss to dairy farms and reduces the health and welfare of dairy cows (Krause and Oetzel, 2006; Plaizier et al., 2012); SARA has been associated with systemic inflammation (Krause and Oetzel, 2006) and dysbiosis of the rumen (Tun et al., 2020) and hindgut microbiota at the luminal, epimural, and mucosa associated levels (Plaizier et al., 2021). Such dysbiosis changes the composition and reduces the functionality of rumen and hindgut microbiota by promoting proliferation of opportunistic bacteria (e.g., faster growers) and disintegration and lysis of those that cannot adapt to suboptimal rumen or

hindgut environments (e.g., pH; Khafipour et al., 2016; Tun et al., 2020). As a result, endotoxins, such as LPS from gram-negative bacteria (Li et al., 2012; Khafipour et al., 2016), and perhaps lipoteichoic acid (LTA) from gram-positive bacteria (Weber et al., 2003), are released into the ruminal and intestinal digesta. These releases increase the concentration of luminal endotoxins and contribute to gut epithelial damage (Chin et al., 2006), particularly of the rumen papillae (Steele et al., 2009). The severity and frequency of SARA episodes dictate the magnitude of epithelial damage and the level of infiltration of immune cells, such as neutrophils and monocytes, in the rumen and intestinal tissues (Plaizier et al., 2012; Abbas et al., 2016; Khiaosa-ard and Zebeli, 2018). They also trigger release of reactive oxygen species and their catalyzers, such as myeloperoxidase (MPO) from these cells (Arroyo et al., 2017; Chami et al., 2018; Zhao et al., 2018). The combination of these events, accompanied by translocation of endotoxins and other immunogenic compounds out of the digestive tract and into peripheral blood circulation, may result in a systemic acute-phase protein (APP) response (Ginsburg, 2002; Eckel and Ametaj, 2016). This response may be driven by the release of proinflammatory cytokines, such as IL-1 β and IL-6 (Trevisi et al., 2015). Hence, strategies that help stabilize conditions across the gastrointestinal tract, attenuate endotoxin release during SARA, prime the functionality of immune cells, and strengthen immune response may mitigate the adverse effects of SARA on health and production of lactating dairy cows.

A rich body of evidence suggests that SCFP supplementation is one such strategy. It has been demonstrated that SCFP stabilize the rumen microbial community (Tun et al., 2020), tend to reduce ruminal free LPS concentration, and enhance immune responses within and outside of the gut (Jensen et al., 2008; Perdomo Lozada, 2011; Li et al., 2012;

Vailati-Riboni et al., 2021). As a result, we hypothesized that gut disruptions and inflammation during SARA can be attenuated by SCFP supplementation. The objectives of the study were, therefore, to determine the effects of 2 commercially available SCFP (XPC and NutriTek, Diamond V) on concentrations of LPS in the rumen and feces, concentrations of LPS and LTA of peripheral plasma, rumen papillae inflammatory status as determined by its MPO content, and systemic APP and cytokines responses of Holstein dairy cows subjected to 2 episodes of grain-induced SARA challenge during early lactation.

3.3 Materials and Methods

This experiment was part of a larger randomized block design experiment conducted over 16 wk from 4 wk before to 12 wk after parturition. The protocol for the use of experimental animals was approved by the University of Manitoba Animal Care Committee (Winnipeg, Canada; protocol no. F14-038) and followed the guidelines of the Canadian Council for Animal Care (CCAC, 1993). Duration of ruminal pH below 5.6 from previous SCFP studies was used for power calculation. With a power of 0.80 and a type 1 error of 0.05, a minimum of 6 replicates per treatment was required to identify a significant reduction in duration of ruminal pH below 5.6 as a result of SCFP supplementation. To account for potential loss of cows during the transition period, 8 replicates per treatment were included in the experiment. Here, we describe a subset of data on endotoxin and inflammatory responses during SARA challenges. Rumen production parameters and ruminal and total-tract digestibility data during SARA challenges are presented in Khalouei et al. (2021).

3.3.1 Animals and diets

A total of 32 parity-2 ($n = 16$) and multiparous ($n = 16$) clinically healthy dairy cows were assigned into a randomized complete block design that contained 8 blocks. Cows were blocked based on parity, milk yield, and calving date. All cows were fixed with rumen cannulas at approximately 12 wk before their expected calving date and were fully recovered from surgery before the start of the study, as evaluated by veterinarian staff. Cows within each block were randomly assigned to 1 of 4 treatments: with no supplementation (control), or supplemented with 14 g/d Diamond V Original XPC (SCFPa, Diamond V), 19 g/d NutriTek (SCFPb-1X, Diamond V), or 38 g/d NutriTek (SCFPb-2X, Diamond V) mixed with 140, 126, 121, and 102 g/d of ground corn, respectively. The treatment diets were implemented from 4 wk before until 12 wk after parturition. The cows were fed individually, and SCFP was supplemented once daily as a top-dress.

3.3.2 Experimental design

Cows were fed a prepartum diet with assigned treatment supplementation from the 4 wk prior to calving until parturition (Table 3-1). Following parturition, cows were fed the lactation diet containing 45% concentrate (DM basis) and their assigned treatment supplementation, except when SARA was induced. Grain-based SARA challenges were conducted during wk 5 (SARA1) and wk 8 (SARA2) of lactation by replacing 20% of the DM of the lactation TMR with pellets containing 50% ground wheat and 50% ground barley. Pellets were mixed with the rest of the TMR, and this replacement was completed

gradually over 3 d before the beginning of SARA1 and SARA2. Detailed descriptions of feed sample collection and analyses are reported in our companion manuscript (Khalouei et al., 2021). The chemical and nutrient compositions of diets are summarized in Table 1. During a 2-wk washout period between the 2 SARA challenges, cows were switched back to the lactation diet with treatment supplementation. Cows were fed the appropriate TMR *ad libitum* once daily at 0900 h, with feed refusal allowance of 5% to 10%. Cows had free access to fresh water during the experimental period. Rumen fluid, feces, and blood samples were collected once during the weeks of Pre-SARA1 (wk 4), Post-SARA1 (wk 7), and Post-SARA2 (blood samples on wk 10 and rumen and feces samples on wk 12) stages. During the SARA1 (wk 5) and SARA2 (wk 8) stages, samples were collected on the second and fifth day of the week (time points indicated as SARA1.1, SARA1.2, SARA2.1, and SARA2.2). All samples were collected 6 h after the 0900 h feed delivery. A schematic of the experimental design and sample collection is shown in Figure 3-1.

3.3.3 Sample collection and analyses

Sample Collection. Approximately 500 mL of whole rumen contents were collected into a 500-mL bottle from 5 sites (cranial, caudal, dorsal, caudal ventral, and caudal dorsal) within the rumen and mixed. Solid and liquid digesta were separated using a Bodum coffee filter plunger (Bodum Inc.), as described by Petri et al. (2013). Briefly, 40 mL of rumen fluid was transferred into 50-mL sterile centrifuge tubes and kept on ice until centrifugation at $12,000 \times g$ for 40 min. Subsequently, the supernatant was filtered through a 0.22- μ m LPS-free filter (Sigma, cat. no. Z359904) into a glass tube and baked at 100°C for 30 min (Li et al., 2012). Samples were then stored at -20°C for further LPS

and LTA analyses.

Table 3-1. Ingredient and chemical compositions of experimental diets

Item	Close-up dry cow TMR	Lactating TMR	SARA TMR
Ingredient composition (% of DM)			
Round bale mixed alfalfa /grass silage	20	35	28
Alfalfa 1 st cut haylage	8	-	-
Corn silage	37	-	-
Barley silage	-	20	16
Straw	13	-	-
Ground corn	-	20	16
Dairy Aide ¹	-	25	20
Close-up dry cow supplement ²	22	-	-
Wheat-Barley pellets	-	-	20
Chemical composition (% of DM)			
DM (%)	48	51	60
CP	15.5	17.9	17.2
Fat	2.8	4.3	3.6
Starch	17.6	18.6	27.9
NDF	38.7	34.9	28.2
ADF	26.7	26.0	19.9
Ca	1.26	1.32	1.06
P	0.37	0.45	0.47
Mg	0.41	0.37	0.34
Na	0.06	0.33	0.27
K	2.37	2.57	2.02

¹Dairy Aide contained: Corn – Flaked (65.2%), Corn Distillers grain (7.1%), APF Fat Plus (3.6%), Feather Meal (3.6%), Porcine Meat Meal (2.9%), Soybean Meal (2.9 %), Canola Meal (2.9%), AV Fat Rothsay Feeders Choice (2.2%), sodium sesquicarbonate (SQ 810, 1.9%), Dicalcium Phosphate (1.5%), potassium chloride (DYNA K Red, 1.5%), Ground Limestone (1.5), Salt – Potash (1.5%), Dairy LMK Ultra micro (0.6 %), Magnesium Oxide (0.3%), Methionine Analogue – Novus (0.3%)

² Dry cow supplement contained Landmark close-up dry cow pellets (50.0%), flaked corn (20.0%), beet pulp pellets (15.0 %), rolled barley (12.5%), liquid molasses (2%), soy oil (0.5%), liquid caramel (0.01%) . Landmark close-up day cow pellets contained: barley (4.0%), limestone(7.0%), corn distillers grain (34.9%), dicalcium phosphate (1.8%), canola meal (23.0%), soybean meal (8.5%), wheat (15.0%), niacin (0.3%), biopowder SXC (0,05%), magnesium oxide (1.6%), transition VB 25K (2.9%), dry cow microPX premix (1%).

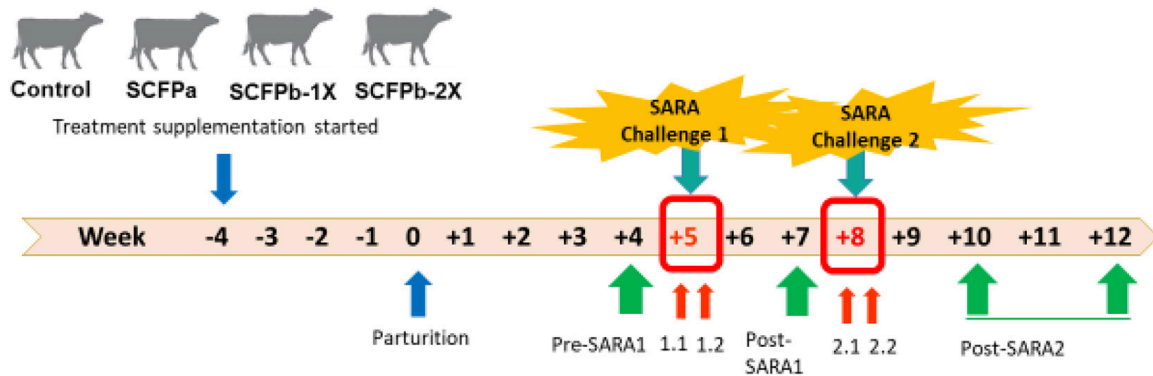


Figure 3-1. Experimental design. A total of 32 cows were randomly divided into 4 treatment groups with 8 cows per group based on parity, level of production, and calving date. Treatments were as follows: control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek (Diamond V) mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn. SARA was induced during wk 5 (SARA1) and 8 (SARA2) post-parturition. Week 4 was considered as Pre-SARA1, wk 7 as Post-SARA1, and wk 10 or 12 (depending on the parameter) as Post-SARA2. Samples were collected weekly, with the exception of challenge periods, during which sampling was performed on d 2 and 5 of each SARA challenge week (SARA1.1, SARA1.2, SARA2.1, and SARA2.2).

Approximately 40 g of fecal grab samples were collected from the rectum after cleaning of the perineal area, placed into 50-mL sterile centrifuge tubes, and kept on ice. Briefly, 20 g of feces was mixed with equal amounts of physiological saline (0.90% wt/vol of NaCl), centrifuged, and processed as described previously for rumen samples. Filtered fecal samples were also kept at -20°C for further LPS analysis.

To obtain papillae samples, rumens were partially emptied of their digesta during wk 4 and wk 10. Papillae samples were then clipped from 2 to 3 sites of the ventral sac, using a sterilized scissor. Samples were washed 10 times immediately after collection with ice-cold PBS (pH 7.4) to remove the residual feed and gastrointestinal tract contents (Steele

et al., 2009). Papillae samples (100 mg) were transferred into prelabeled 2-mL vials, snap-frozen in liquid nitrogen, and stored at -80°C for MPO detection.

Blood samples were taken at 6 h after feeding (1500 h) from the tail vein in plasma heparinized (143 US Pharmacopeia units) and blank serum evacuated tubes (Thermo Fisher Scientific). Briefly, 10 mL of sample in serum tubes were kept at room temperature for 30 min before centrifuging at $3,000 \times g$ at 4°C for 15 min; from this, 1 mL of serum was stored at -20°C for APP (haptoglobin, Hp) and cytokine (IL-6 and IL- 1β) analyses. The set of 10-mL blood samples in plasma tubes were immediately centrifuged at $3,000 \times g$ at 4°C for 15 min. Subsequently, 1 mL of plasma was transferred into pyrogen-free glass tubes and stored at -20°C for LPS and LTA analyses. Another 1 mL of plasma was stored at -20°C for APP serum amyloid A (SAA) and LPS-binding protein (LBP) measurements.

LPS and LTA Analyses. Concentrations of LPS in rumen fluid and feces were determined by a chromogenic kinetic Limulus ameobocyte lysate assay (Kinetic-QCL, cat. no. 50-650u) using a 50% β -glucan blocker (cat. no. N190; both from Lonza Group Ltd.) as previously described (Khafipour et al., 2009b). Blood plasma, rumen fluid, and feces were diluted with Limulus ameobocyte lysate reagent water (cat. no. W50-500), 10 mM MgCl solution (cat. no. S50-641), and Pyrosperser (cat. no. N188; all from Lonza Group Ltd.). Dilution rates were 1:80,943 for rumen fluid, 1:50,719 for feces, and 1:28 for blood plasma. To verify the lack of LPS inhibition, samples were spiked with 3 μL of 5 endotoxin units (EU)/mL commercial *Escherichia coli* LPS standard (Lonza Group Ltd.). A recovery range between 50 and 150% of the spiked concentration was considered as noninhibitory dilution, as suggested by the manufacturer. As our recovery rates were

83.3% $\pm$ 0.04% (mean $\pm$ SD) for rumen liquid and 92.4% $\pm$ 0.03% for feces, the kits performed satisfactorily.

The concentrations of LTA in rumen liquid and blood plasma were determined using an ELISA assay with a commercial kit (OKEH02591, Aviva Systems Biology) according to manufacturer's instructions. Rumen liquid samples were diluted from 0 to 10⁻⁶; blood plasma samples were analyzed without dilution. Optical densities were determined at 405 nm for the LPS assay and 450 nm for the LTA assay, as per kits' instructions, using a microplate spectrophotometer (Synergy H4 System, BioTek Instruments Inc.).

Measures of Inflammatory Response. For measurement of SAA and LBP, blood plasma samples were diluted 1:500 with distilled water. Diluted samples were then processed using a commercial ELISA kit for SAA (cat. no. TP802, Tridelata Development Ltd.) and LBP (cat. no. HK503, Hycult Biotech), as described by Khafipour et al. (2009b). The Hp concentration in blood serum was determined with a commercial ELISA kit (cat. no. TP801, Tridelata Development Ltd.) following the manufacturer's instructions. All optical densities were determined using the recommended wavelengths by the kit manufacturers, using a microplate spectrophotometer (Synergy H4 System, BioTek Instruments Inc.). The recommended wavelengths were 450 nm for SAA and LBP and 630 nm for Hp. Optical densities from negative controls and 6 dilutions of positive controls were used for development of a standard curve and calculation of SAA, LBP, and HP in samples.

Serum IL-6 and IL-1 β concentrations were determined with a Bovine IL-6 ELISA Reagent Kit (ESS0029) and a Bovine IL-1 β ELISA Reagent Kit (ESS0027; both from Thermo Fisher Scientific), respectively. Additional ELISA reagent kit buffers used

included DPBS (28374), carbonate-bicarbonate buffer (28382), blocking buffer (N502), wash buffer (N503), and BSA (A4737-1G; all from Thermo Fisher Scientific). Optical densities for IL-6 and IL-1 β assays were read under recommended 450-nm wavelengths using a microplate spectrophotometer (Synergy H4 System, BioTek Instruments Inc.). The recovery range was 82 to 93% for IL-6 and 96 to 116% for IL-1 β , respectively.

Myeloperoxidase Concentration in Rumen Papillae. Frozen papillae samples that had been stored at -80°C were ground in liquid nitrogen with a mortar. Briefly, 50 mg of ground samples were transferred into 2-mL centrifuge tubes with 1 mL of fresh lysis buffer (IS007-BUF3, Cloud-Clone Corp.) and sonicated with an ultrasonic processor (VC 130, Sonics & Materials Inc.). Subsequently, MPO concentration was determined using a Bovine MPO ELISA kit (SEA601Bo, Cloud-Clone Corp.) according to the manufacturer's instructions. Optical densities were read immediately at 450 nm using a microplate spectrophotometer (Synergy H4 System, BioTek Instruments Inc.). The recovery range was 80 to 102%. Total protein concentration was determined using a Pierce BCA protein assay kit (SF249046, Thermo Fisher Scientific). The MPO concentration was expressed as nanograms of total protein.

3.3.4 Statistical analysis

Normality of distributions of residuals for LPS, LTA, APP, and cytokine data was tested using the UNIVARIATE procedure of SAS (version 9.4, SAS Institute Inc.) by Shapiro-Wilk's statistic. If the residuals were not normal, the data were either log₁₀ transformed or raised to the power of lambda to achieve normality (Tables 2, 3, 4, and 5). Lambda was calculated by Box-Cox transformation using the TRANSREG procedure of

SAS. Original or transformed data were then analyzed by the MIXED procedure of SAS using the following model:

$$Y_{ijk} = \mu + T_i + S_j + P_k + (T \times S)_{ij} + (T \times P)_{ik} + (P \times S)_{kj} + (T \times P \times S)_{ijk} + e_{ijk}.$$

The variable Y_{ijk} was dependent on μ as the average experimental value and fixed effects of treatment T_i (i = control, SCFPa, SCFPb-1X, and SCFPb-2X), stage S_j (j = Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2), parity P_k (k = parity 2 or 3+), and interactions $(T \times S)_{ij}$, $(T \times P)_{ik}$, $(P \times S)_{kj}$, and $(T \times S \times P)_{ijk}$. Data from the second and fifth days of each SARA week (time points SARA1.1, SARA1.2, SARA2.1, and SARA2.2) were combined as SARA1 and SARA2, as no difference was observed between SARA1.1 and SARA1.2 or between SARA2.1 and SARA2.2. Level of LTA was measured only during SARA1, and SARA1.1 and SARA1.2 data were presented separately. Block was considered as random, and stage was considered as a repeated measure. The PDIFF option was applied for pairwise comparisons between treatments, parities, stages, and their interactions. Comparison of least squares means was adjusted with the Tukey test. Contrast comparisons were made between control and SCFP, SCFPa and SCFPb, and SCFPb-1X and SCFPb-2X. All analyses were initially performed with the MIXED model described herein. If the effect of parity was not significant or no trend was observed, parity and its interactions were removed from the model (Tables 2, 3, 4, and 5).

Results are presented as least squares means with their pooled standard errors. For results interpretation, the data that were transformed to achieve normality are presented as means from the original data within tables (Tables 2, 3, 4, and 5). Significant effects were considered at $P < 0.05$, and tendencies were discussed at $0.05 \leq P < 0.1$.

3.4 Results

The health of animals was monitored daily. During the larger study 2 cows died, one from the SCFPb-2X group, which died during the prepartum period due to complications of cannulation surgery, and the other from the SCFPb-1X group, which died following the first SARA challenge. Samples from these cows were removed from the data set. Additionally, one cow in the SCFPa group during the transition period and one cow in SCFPb-1X after the second SARA challenge were diagnosed with mastitis. Also, one cow in the SCFPb-2X group was diagnosed with milk fever during the transition period; however, she recovered before SARA1 was conducted. Data from these cows during the indicated time points were removed from the statistical analysis.

3.4.1 Effects of SARA on bacterial endotoxin (LPS and LTA) concentrations in the rumen, feces, and plasma

Pooled across SCFP treatments, the SARA challenges compared with pre- and post-challenge stages increased the concentration of free LPS in the rumen fluid (66,140 vs. 5,215 EU/mL, $P < 0.001$) and in the feces (81,628 vs. 8,221 EU/mL, $P < 0.01$; Table 3-2). The free LPS concentrations in rumen fluid and feces did not differ between the 2 SARA challenge stages, or among the Pre-SARA1, Post-SARA1, and Post-SARA2 stages (Table 3-2). The LPS concentrations in the plasma samples were below the detection limit of the assay (<0.005 EU/mL) during all stages. The SARA challenges tended to increase ($P = 0.07$) plasma LTA concentrations compared with Pre-SARA1 (0.70 vs. 0.65 ng/mL; Table 3-3).

Table 3-2. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) and parity on rumen and feces free lipopolysaccharide (LPS) concentrations of lactating dairy cows

Item	Treatment ¹				Stage ²					SEM	P- values ³						Contrasts ⁴		
	Control	SCFPa	SCFPb-1X	SCFPb-2X	Pre-SARA1	SARA1	Post-SARA1 (wk 7)	SARA2	Post-SARA2 (wk 12)		Treat	Stage	Parity	Treat × Stage	Treat × Parity	Stage × Parity	1	2	3
Rumen LPS, (EU/mL) ⁵	15,845	14,791	15,488	12,022	5,012 ^y	63,096 ^x	6,166 ^y	69,183 ^x	4,467 ^y	2,452	0.37	<0.001	0.06	0.93	0.06	0.04			
Parity 2	13,182 ^A	12,022	13,183	6,670 ^B	3,548 ^y	56,234 ^x	4,677 ^{yX}	64,565 ^x	2,512 ^{yYn}	1,779	0.002	<0.001	-	0.13	-	-	0.39	0.57	0.13
Parity 3+	18,197	22,387	19,055	20,417	7,244 ^y	74,131 ^x	8,710 ^y	79,433 ^x	8,709 ^{ym}	3,622	0.93	<0.001	-	0.91	-	-			
Fecal LPS, (EU/mL)	16,982	20,893	19,953	23,988	10,471 ^y	74,131 ^x	7,586 ^y	89,125 ^x	6,606 ^y	3,642	0.43	<0.001	-	0.38	-	-	0.14	0.79	0.41

^{A,B}Means in a row with different superscripts among Treatments tended to be different ($0.05 \leq P < 0.1$).

^{x,y}Means in a row with different superscripts among Stages are different ($P < 0.05$).

^{x,y}Means in a row with different superscripts among Stages tended to be different ($0.05 \leq P < 0.1$).

^{m,n}Means in a column with different superscripts between Parities are different ($P < 0.05$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) after parturition. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 12 as Post-SARA2.

³Statistical analyses were conducted on log₁₀-transformed data.

⁴Contrasts: 1 = Control vs. SCFP, 2 = SCFPa vs. SCFPb, 3 = SCFPb-1X vs. SCFPb-2X.

⁵EU = Endotoxin unit.

Table 3-3. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1.1, and SARA1.2) and parity on lipoteichoic acid (LTA) concentration in blood plasma of lactating dairy cows

Item	Treatment ¹				Stage ²			Parity			P- values ³								
	Control	SCFPa	SCFPb-1X	SCFPb-2X	Pre-SARA1	SARA1.1	SARA1.2	2	3	SEM	Treat	Stage	Parity	Treat × Stage	Treat × Parity	Stage × Parity	Contrasts ⁴		
																	1	2	3
LTA ⁵ , (ng/mL)	0.73 ^{aA}	0.71 ^{ab}	0.63 ^b	0.65 ^{abB}	0.65 ^Y	0.70 ^X	0.69 ^{XY}	0.72 ^m	0.64 ⁿ	0.03	0.006	0.07	0.03	0.95	0.69	0.57	<0.01	0.02	0.49

^{a,b}Means in a row with different superscripts among Treatments are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts among Treatments tended to be different ($0.05 \leq P < 0.1$).

^{m,n}Means in a row with different superscripts between Parities are different ($P < 0.05$).

^{X,Y}Means in a row with different superscripts among Stages tended to be different ($0.05 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: The first SARA was induced during weeks 5 after parturition. Week 4 was considered as Pre-SARA1, day 2 on week 5 as SARA1.1, and day 5 on week 5 as SARA1.2.

³Statistical analyses were conducted on original values without any transformation.

⁴Contrasts: 1 = Control vs. SCFP, 2 = SCFPa vs. SCFPb, 3 = SCFPb-1X vs. SCFPb-2X.

⁵LTA= Lipoteichoic acid.

3.4.2 Effects of SCFP on bacterial endotoxin (LPS and LTA) concentrations in the rumen, feces, and plasma

The SCFP treatments did not affect ruminal and fecal free LPS concentrations before, after, or during SARA challenges. However, parity and interaction of parity and SCFP treatments tended to affect ($P = 0.06$) rumen free LPS (Table 3-2). In parity-2 cows, pooled across Pre-SARA, Post-SARA, and SARA stages, SCFPb-2X tended to reduce ($P = 0.077$) rumen free LPS concentrations compared with control (6,670 vs. 13,182 EU/mL). The LPS concentrations in plasma samples remained undetected for all treatments.

The concentration of LTA was affected by SCFP treatment ($P = 0.006$) and parity ($P = 0.03$), but not by any of the interactions (Table 3-3). Pooled across Pre-SARA1 stage and SARA challenge stages, the SCFPb-1X treatment reduced ($P = 0.006$) and SCFPb-2X tended to reduce ($P = 0.07$) plasma concentrations of LTA compared with control (0.63 and 0.65 vs. 0.73 ng/mL, respectively). Contrast comparisons showed a reduced plasma LTA in SCFP compared with control (0.66 vs. 0.73 ng/mL; $P = 0.008$) and in SCFPb compared with SCFPa (0.64 vs. 0.71 ng/mL; $P = 0.02$). Average across SCFP treatments and SARA stages, parity-2 cows had greater ($P = 0.03$) plasma LTA compared with parity-3+ cows (0.72 vs. 0.64 ng/mL).

3.4.3 Effects of SARA on inflammatory responses

Regardless of SCFP treatments, the SARA challenges increased ($P < 0.01$) the blood plasma concentrations of SAA compared with Pre-SARA1 (63.38 vs. 19.76 $\mu\text{g/mL}$) but did not affect that of Hp (129.03 vs. 81.61 $\mu\text{g/mL}$) or LBP (11.86 vs. 11.16 $\mu\text{g/mL}$; Table

3-4). Compared with SARA1, plasma LBP concentration tended to be lower ($P = 0.07$) during Post-SARA1 (10.01 vs. 12.77 $\mu\text{g/mL}$) and was reduced ($P < 0.01$) during Post-SARA2 (8.99 vs. 12.77 $\mu\text{g/mL}$). The SARA challenges did not affect the blood plasma concentration of cytokine IL-6, regardless of SCFP treatments (Table 3-4). Pooled across SCFP treatments, plasma IL-1 β concentration tended to increase ($P = 0.07$) from Pre-SARA1 to Post-SARA2 (4.51 vs. 8.71 pg/mL). We found no difference between the 2 SARA challenges in all parameters. The MPO concentration in rumen papillae did not differ between the Pre-SARA1 and Post-SARA2 stages (Table 3-5).

3.4.4 Effects of SCFP on inflammatory responses

Pooled across Pre-SARA, Post-SARA, and SARA stages, SCFP treatments affected the blood plasma concentrations of SAA and LBP, and blood serum concentrations of Hp, IL-1 β , and IL-6 (Table 3-4). The interaction between stage and SCFP treatments was not significant for any of these variables. Feeding SCFP resulted in reduced ($P = 0.02$) plasma SAA concentrations compared with the control group (27.87 vs. 39.81 $\mu\text{g/mL}$). This reduction was more pronounced in SCFPb-2X cows compared with control cows (23.14 vs. 39.81 $\mu\text{g/mL}$; $P = 0.05$). Supplementation with SCFPb reduced serum Hp concentrations compared with SCFPa (90.51 vs. 141.74 $\mu\text{g/mL}$; $P = 0.05$). Between SCFPb treatments, SCFPb-2X resulted in more reduction ($P = 0.03$) in Hp compared with SCFPb-1X (63.30 vs. 117.73 $\mu\text{g/mL}$). Regardless of SCFP type, SCFP feeding tended to reduce ($P = 0.09$) plasma LBP compared with control (10.40 vs. 11.68 $\mu\text{g/mL}$). In particular, SCFPa supplementation reduced plasma LBP concentrations compared with control (9.22 vs. 11.68 $\mu\text{g/mL}$; $P = 0.05$) and SCFPb (9.22 vs. 11.04 $\mu\text{g/mL}$; $P = 0.03$).

Table 3-4. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) and parity on peripheral blood acute phase proteins and cytokines concentrations of lactating dairy cows

Item ⁵	Treatment ¹				Stage ²						SEM	P- values ³							
	Control	SCFPa	SCFPb-1X	SCFPb-2X	Pre-SARA1	SARA1	Post-SARA1 (wk 7)	SARA2	Post-SARA2 (wk 10)	Treat		Stage	Parity	Treat × Stage	Treat × Parity	Stage × Parity	Contrasts ⁴		
																	1	2	3
Acute Phase Proteins																			
SAA, (µg/mL)	39.81 ^a	32.90 ^{ab}	27.57 ^{ab}	23.14 ^b	19.76 ^y	71.08 ^x	21.32 ^y	55.68 ^x	15.15 ^y	5.18	0.05	< 0.001	-	0.59	-	-	0.02	0.11	0.41
Hp, (µg/mL)	89.54 ^{ab}	141.74 ^a	117.73 ^{ab}	63.30 ^b	81.61	132.56	68.53	125.50	100.26	29.68	0.04	0.11	-	0.49	-	-	0.53	0.05	0.03
LBP, (µg/mL)	11.68 ^a	9.22 ^b	11.03 ^{ab}	11.05 ^{ab}	11.16 ^{xy}	12.77 ^{xX}	10.01 ^{xyY}	10.95 ^{xy}	8.99 ^y	1.08	0.05	< 0.001	0.19	0.59	0.001	0.22			
Parity 2	11.23	9.01	8.73	8.36	9.22	11.85	8.75	10.17	7.05	1.04	0.11	0.006	-	0.90	-	-	0.09	0.03	0.98
Parity 3+	12.21 ^{abA}	9.18 ^{bB}	14.16 ^a	15.18 ^a	13.01	13.02	10.83	11.15	11.05	1.18	0.001	0.29	-	0.19	-	-			
Cytokines																			
IL-1β (pg/mL)	11.94 ^a	4.13 ^b	9.45 ^a	3.71 ^b	4.51 ^Y	6.09 ^{XY}	6.05 ^{XY}	7.69 ^{XY}	8.71 ^X	1.89	< 0.001	0.04	< 0.001	0.71	< 0.001	0.79			
Parity 2	19.35 ^a	5.13 ^b	28.99 ^{am}	16.45 ^{am}	10.69	14.09	15.62	17.18	17.30	2.77	<0.001	0.48	-	0.78	-	-	< 0.001	0.03	< 0.001
Parity 3+	7.37 ^a	3.32 ^b	3.08 ^{bn}	0.84 ^{cn}	1.91	2.63	2.35	3.44	4.39	2.03	<0.001	0.04	-	0.89	-	-			
IL-6 (ng/mL)	338.86 ^{ab}	256.39 ^{bB}	419.52 ^{aA}	442.53 ^a	304.48	357.99	363.43	391.39	370.74	75.78	0.01	0.83	< 0.001	0.99	0.02	0.99			
Parity 2	466.94 ^{bc}	352.13 ^c	887.76 ^{am}	837.06 ^{abm}	493.88	598.36	631.63	663.28	583.53	165.81	<0.001	0.82	-	0.99	-	-	0.62	0.001	0.78
Parity 3+	245.92	186.66	198.24 ⁿ	233.95 ⁿ	187.69	214.18	209.12	230.93	235.54	64.31	0.50	0.93	-	0.99	-	-			

^{a-c}Means in a row with different superscripts among Treatments are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts among Treatments tended to be different ($0.05 \leq P < 0.1$).

^{x,y}Means in a row with different superscripts among Stages are different ($P < 0.05$).

^{X,Y}Means in a row with different superscripts among Stages tended to be different ($0.05 \leq P < 0.1$).

^{m,n}Means in a column with different superscripts between Parities are different ($P < 0.05$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on ln-transformed data. Presented means are original values prior to transformation.

⁴Contrasts: 1 = Control vs. SCFP, 2 = SCFPa vs. SCFPb, 3 = SCFPb-1X vs. SCFPb-2X.

⁵SAA = Serum amyloid A; Hp = Haptoglobin; LBP = Lipopolysaccharide (LPS)-binding protein; IL-1β = Interleukin-1β; IL-6 = Interleukin-6

The effects of SCFPa compared with SCFPb were more pronounced in parity-3+ cows compared with parity-2 cows.

Pooled across Pre-SARA, Post-SARA, and SARA stages, SCFP supplementation reduced ($P < 0.001$) blood serum IL-1 β concentrations compared with control (5.25 vs. 11.94 pg/mL). The reduction in serum IL-1 β concentrations was greater in SCFPa than SCFPb (4.13 vs. 6.58 pg/mL; $P = 0.03$) and in SCFPb-2X compared with SCFPb-1X (3.71 vs. 9.45 pg/mL; $P < 0.001$). Similar to IL-1 β , SCFPa-fed cows had lower ($P = 0.03$) serum IL-6 concentration compared with SCFPb cows (256.39 vs. 431.02 ng/mL). Average across SCFP treatments and SARA stages, parity-2 cows had greater ($P < 0.01$) serum IL-1 β (17.48 vs. 3.65 pg/mL) and IL-6 concentrations (635.97 vs. 216.19 pg/mL) compared with parity-3+ cows.

The MPO concentration in rumen papillae did not differ between the Pre-SARA1 and Post-SARA2 stages or among SCFP treatments (Table 3-5).

Table 3-5. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and sampling periods (Pre-SARA1, Post-SARA2) on myeloperoxidase (MPO) concentrations (ng/ μ g total protein) in rumen papillae of lactating dairy cows

Item	Treatment ¹				Stage ²		SEM	P-value ³					
	Control	SCFPa	SCFPb-	SCFPb-	Pre-SARA1 (wk 4)	Post-SARA2 (wk 10)		Treat	Stage	Treat × Stage	Contrasts ⁴		
			1X	2X							1	2	3
MPO ⁵ , (ng/μg)	1.16	1.15	1.12	1.00	1.11	1.11	0.07	0.37	0.97	0.94	0.28	0.22	0.19

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: Week 4 was considered as Pre-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on original values without any transformation.

⁴Contrasts: 1 = Control vs. SCFP, 2 = SCFPa vs. SCFPb, 3 = SCFPb-1X vs. SCFPb-2X.

⁵MPO: Myeloperoxidase.

3.5 Discussion

Postbiotics from *Saccharomyces cerevisiae* fermentation contain a range of bioactive compounds that stabilize rumen microbiota and reduce inflammatory response, resulting in improved gut health and immune balance (Jensen et al., 2008; Li et al., 2016; Tun et al., 2020). In this study, we tested whether 2 commercially available SCFP (SCFPa, and SCFPb at 1X and 2X doses) can ameliorate the disrupted gut and immune health of dairy cows subjected to SARA challenges. We demonstrated that supplementation of SCFPb at the 2X dose attenuated free LPS concentration in the rumen fluid in parity-2 cows. We further showed that supplementation of SCFPb at both 1X and 2X doses attenuated plasma concentration of LTA. This perhaps was an indicator of either a reduced translocation of LTA endotoxins from the gut lumen and, hence, improved gut epithelial integrity, or greater capacity of the immune system for clearing LTA from the systemic circulation. Also, SCFPb supplementation at the 2X dose reduced plasma concentration of SAA and IL-1 β compared with control-fed cows, indicating a reduced proinflammatory status, perhaps due to a priming effect of SCFP. Additionally, SCFPb supplementation showed more effect on inflammatory parameters compared with SCFPa, which could be due to its higher content of antioxidants and polyphenol compounds (Khalouei et al., 2021).

3.5.1 Assessment of SARA Induction Protocol

In our study, SARA was defined as daily episodes of rumen pH between 5.2 and 5.6 for at least 180 min per day (Gozho et al., 2007). These thresholds are important, in that at least 3 h per day of pH below 5.6 is required to provoke an inflammatory response

(Gozho et al., 2007). The companion paper from Khalouei et al. (2021) reported that, across treatments, the SARA challenge increased the duration of rumen pH below 5.6 from 8 to 186 min/d. Hence, on average, the duration of rumen pH below 5.6 during the SARA challenge was only 6 min/d longer than the threshold. As this duration did not greatly exceeded the SARA threshold, it was expected that a less severe inflammatory response or gut disturbance would be observed, compared with previously reported grain-induced SARA challenges with durations longer than the threshold (e.g., 100 min longer in Khafipour et al., 2009b, or 200 min longer in Zhao et al., 2018).

3.5.2 Effect of SARA on LPS concentrations in rumen fluid and feces

The observed increases in ruminal free LPS concentrations were lower than those reported in the literature during grain-based SARA challenges (e.g., from 24,547 to 128,820 in Gozho et al., 2007; from 30,768 to 130,589 in Zhao et al., 2018; and from 14,581 to 168,285 EU/mL in Humer et al., 2018). This confirms that moderate pH depression in our study resulted in moderate increases in ruminal free LPS. In contrast, the observed increases in fecal free LPS were similar to previously reported increases in the literature (e.g., from 12,832 to 93,154 and from 29,679 to 77,371 EU/g of wet feces in Li et al., 2012, and Li et al., 2016). In contrast, Kumar et al. (2017) reported greater concentrations of free LPS for both baseline and SARA, from 133,165 to 1,049,198 during an abrupt SARA induction similar to our study, and from 175,245 to 1,256,181 EU/g wet feces when SARA was continuously induced for 2 wk. The concentration of free LPS in the feces is a function of carbohydrates bypassed from the rumen to the large

intestine (Li et al., 2012). A survey (Li et al., 2010) of 300 lactating dairy cows on 10 farms reported that fecal free LPS ranged from 3,514 to 252,345 with a median of 34,982 and 75th percentile of 63,179 EU/g of wet feces. In that survey, the variations in fecal free LPS were mostly explained by the amount of dietary NDF among farms, DIM, and plasma Hp concentration among cows. Considering the evidence, the concentrations reported in the study of Kumar et al. (2017) were perhaps on the higher end, and our SARA challenges successfully increased fecal LPS to the ranges that are usually expected during SARA. Additionally, when free ruminal and fecal LPS between the 2 SARA challenges were compared, SARA1 and SARA2 did not differ in these LPS contents, confirming the consistency of our induction model (Table 3-2; Table 3-6). Also, a lack of difference in ruminal or fecal free LPS contents among Pre-SARA1, Post-SARA1, and Post-SARA2 was indicative that cows returned to the baseline (Pre-SARA1) within 2 to 4 wk after SARA challenge.

3.5.3 Effect of SCFP on LPS concentrations in rumen fluid and feces

Within parity-2 cows, SCFPb-2X supplementation tended to reduce ruminal free LPS concentrations compared with the control group ($P = 0.077$) for both the Pre-SARA1 (1,862 EU/mL) and pooled across SARA weeks (46,707 EU/mL; Table 3-2; Table 3-7). The rate of LPS release from gram-negative bacteria is a function of availability of nutrients and pH (Hurley, 1995). The availability of highly degradable nutrients in the rumen promotes proliferation of bacteria and, hence, release of LPS during the logarithmic and stationary phases of bacterial growth, while bacteria are expanding their cell walls. Suboptimal rumen pH, however, results in disintegration and lysis of bacterial

cell walls and release of LPS from organisms that cannot tolerate the lower pH (Plaizier et al., 2012; Khafipour et al., 2016; Monteiro and Faciola, 2020). A reduction of ruminal free LPS concentration hence may be an indicator that SCFPb-2X stabilized the rumen microbial community by controlled proliferation of gram-negative bacterial taxa, such as members of Proteobacteria and Bacteroidetes phyla, which are major contributors to the rumen free LPS pool (Mao et al., 2013; Khafipour et al., 2016; Guo et al., 2018). This hypothesis is supported by ruminal microbiome data showing that SCFPb-2X promoted microbiome stability by ameliorating the negative effects of SARA on community evenness and abundances of foundation members (Guo et al., 2018). The SCFPb-2X treatment also stabilized the rumen pH environment (79.3 min/d of pH <5.6) by switching the site of starch digestion from the rumen to the intestines (Khalouei et al., 2021), which perhaps contributed to a lower rumen LPS concentration compared with the control group.

3.5.4 Effects of SARA and SCFP on LPS and LBP concentrations in peripheral blood plasma

Regardless of the high-sensitivity assay used, peripheral plasma LPS concentration remained below our detection limit of 0.005 EU/mL for all samples. This suggests that the rate of LPS translocation through the rumen or hindgut wall was perhaps below the rate of LPS clearance by the liver (Haubro Andersen and Jarlov, 1990). Therefore, LPS did not accumulate in the peripheral plasma above the detection limit of the assay. Plasma LBP concentration also did not differ among Pre-SARA1, SARA1, and SARA 2. The only tendencies or significant differences observed in LBP concentration were among SARA1, Post-SARA1, and Post-SARA2 (Table 3-4, 3-8, 3-9). As LBP is an APP that

only binds to LPS and facilitates its transfer to membrane-associated receptors, its greater concentrations are indicative of LPS trafficking from the gut lumen into circulation (Eckel and Ametaj, 2016). In this study, however, the plasma concentration of LBP during SARA was lower than that of previous reports (e.g., 11.86 vs. 53.2 µg/mL in Khafipour et al., 2009b, or 40.9 µg/mL in Zhao et al., 2018), where authors induced a more severe rumen pH depression below 5.6 (279 and 369 min/d, respectively) compared with current study (186 min/d) and were able to detect LPS in plasma (0.52 and 0.21 EU/mL, respectively).

3.5.5 Effects of SARA and SCFP on LTA concentrations in peripheral blood plasma

The SARA challenge resulted in a tendency for greater LTA on both the second (SARA1.1) and the fifth day (SARA1.2) of SARA compared with that of Pre-SARA1, indicating greater translocation of LTA during the challenge week (Table 3-3, 3-10). That being said, the presence of a detectable amount of LTA in peripheral circulation during Pre-SARA1 (0.65 ng/mL) was interesting and was an indication of weaker immune response and more tolerance to LTA compared with LPS (Eckel and Ametaj, 2016). The weaker immune response of dairy cows to LTA is also evident from tolerance of the udder and the reproductive system to chronic colonization by gram-positive bacteria, compared with a rapid and severe response to colonization/infection by gram-negative bacteria in those organs (Strandberg et al., 2005; Gilbert et al., 2013).

Supplementation with SCFPb-1X significantly reduced plasma concentrations of LTA compared with control cows (0.63 vs. 0.73 ng/mL). We found no difference in plasma LTA concentration among SCFPb-1X, SCFPb-2X, and SCFPa. The reduced LTA

concentration in SCFP-fed cows perhaps is an indicator of either less translocation of LTA endotoxins from the gut lumen and, hence, improved gut epithelial integrity compared with control cows, or a priming of the immune system in those animals (Mahmoud et al., 2020; Vailati-Riboni et al., 2021) for more effective clearance of LTA from circulation. Similar to LPS, LTA has been shown to disrupt barrier function and intestinal epithelial permeability via production of reactive oxygen species, in particular, inducible nitric oxide synthase, which increases the concentration of nitric oxide (Eckel and Ametaj, 2016). We attempted to measure LTA concentration in rumen fluid; however, the results were not reproducible (data not shown). We speculate that perhaps the presence of inhibitors in combination with greater concentrations of LTA in rumen fluid made it difficult to achieve proper dilutions, hence overwhelming the assay. To our knowledge, this is the first study to report LTA under SARA episodes.

3.5.6 Effects of SARA and SCFP on proinflammatory cytokines (IL1- β and IL-6) and APP (SAA and Hp) concentrations in peripheral blood plasma

Both LPS and LTA bind to specific cell surface receptors to activate an endotoxin-associated immune response. Although LPS binds to CD14 and toll-like receptor (TLR) 4, LTA binds to CD14, CD36, and TLR2 to activate the release of proinflammatory mediators such as IL-1 β and IL-6 (Dziarski et al., 2000; Ginsburg, 2002) and, subsequently, the production of acute-phase proteins, such as SAA, Hp, and LBP (Eckel and Ametaj, 2016). In this study, SARA challenges increased blood plasma concentrations of SAA and tended to increase that of IL-1 β compared with Pre-SARA1, without affecting the plasma concentration of Hp and serum concentration of IL-6 (Table 3-4, 3-8, 3-11, 3-12). Consistent with our results, the increase in plasma concentration of

SAA following induction of SARA has been reported previously (Khafipour et al., 2009b; Li et al., 2016; Zhao et al., 2018). Increases in serum concentrations of Hp and IL-1 β were anticipated (Li et al., 2016; Zhao et al., 2018), whereas only a numerical increase or a tendency was observed, which could be because of moderate episodes of rumen pH depression. Although APP usually reach their peak concentration within days post-challenge, cytokine responses occur faster, reaching their peak within several hours post challenge (Jacobsen et al., 2007; Paulina and Tadeusz, 2011). In this study, circulating variables were measured on d 2 and 5 following SARA induction; hence, it is possible that the window for proper monitoring of these cytokines was missed (Jacobsen et al., 2007). For instance, previously, with a more severe SARA induction model (313.5 vs. 186 min/d of rumen pH <5.6) but a similar measurement time point, authors captured a tendency for increased IL-6 during SARA (Li et al., 2016). Therefore, the lack of IL-6 response in the present study may be due to moderate episodes of rumen pH depression. These observations are also backed up by the lack of MPO elevation in rumen papillae, which is an indicator of tissue inflammation (Chami et al., 2018), supporting that, although we met the minimum requirements for induction of SARA, we did not induce a severe inflammation in rumen papillae. We should keep in mind, though, that MPO measurement was performed in papillae samples collected 2 wk after the second SARA challenge. As such, we may have similarly missed the window for proper monitoring of MPO.

From an immunological perspective, we consider an inflammatory response successful if the concentration of proinflammatory cytokines increases rapidly (speed of response) following the challenge and declines soon after achieving a peak (magnitude of

response), to avoid unnecessary damage to the host (Jacobsen et al., 2007; Abbas et al., 2016). However, the magnitude of cytokine production can be reduced if or when immune cells are already primed (or trained) and have greater readiness and capacity to handle the immunogenic compounds or pathogenic organisms that threaten host health (Jacobsen et al., 2007; Vailati-Riboni et al., 2021). Here, across Pre-SARA, Post-SARA, and SARA stages, SCFPb-2X reduced plasma concentration of SAA and IL-1 β and serum concentration of Hp compared with control-fed cows. These effects might be a result of greater priming effect of immune cells by SCFP, as reported previously. For instance, Mahmoud et al. (2020) reported that peripheral blood cells from SCFP-treated calves had an increased capacity for proinflammatory cytokine production in response to LPS stimulation and suggested that this innate training (or priming) has emerged as a mechanism by which cells of the innate immune system exist in an enhanced, or “trained,” state, which enables them to respond to pathogenic and immunogenic insults with increased proinflammatory cytokine secretion.

3.6 Conclusions

Induction of 2 episodes of grain-based SARA challenges during early lactation increased free LPS concentrations in ruminal fluid and feces and tended to increase plasma concentration of LTA. Induction of SARA episodes increased concentration of circulating SAA and tended to increase that of IL-1 β compared with Pre-SARA1, but did not affect the concentrations of circulating IL-6 and Hp. These data, together with undetectable plasma LPS and lack of rumen papillae MPO changes across all Pre-SARA, Post-SARA, and SARA stages, suggest that a moderate depression of rumen pH that

slightly exceeded the SARA threshold promoted an inflammatory response but did not result in epithelial tissue damage. Supplementation of SCFPb at both 1X (19 g/cow per day) and 2X doses attenuated the plasma concentration of LTA. Meanwhile, SCFPb supplementation at the 2X dose (38 g/cow per day) attenuated the free LPS concentrations of ruminal fluid compared with control in parity-2 cows, and also reduced plasma concentration of SAA and serum concentration of IL-1 β compared with control-fed cows. This could perhaps be suggestive of either less translocation of LTA and LPS endotoxins from the gut lumen and, hence, improved gut epithelial integrity associated with SCFPb feeding, or a greater priming of immune cells for more effective clearance of LTA and LPS from the systemic circulation.

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when the study was conducted. He is currently the director of microbiome research at Cargill Health Technologies. The authors have not stated any other conflicts of interest.

Table 3-6. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on rumen and feces free lipopolysaccharide (LPS) concentrations of lactating dairy cows

Item	Treatment ¹	Stage ²					SEM	P- values ³					
		Pre-SARA1	SARA1	Post-SARA1 (wk7)	SARA2	Post-SARA2 (wk12)		Treat	Stage	Parity	Treat× Stage	Treat× Parity	Stage× Parity
Rumen LPS, (EU/mL) ⁴	Control	5,256 ^b	84,236 ^a	6,559 ^b	77,714 ^a	4,207 ^b	3,712						
	SCFPa	4,939 ^b	54,250 ^a	7,517 ^b	68,281 ^a	5,171 ^b	3,537	0.37	<0.001	0.06	0.93	0.06	0.04
	SCFPb-1X	6,123 ^b	58,938 ^a	7,469 ^b	81,245 ^a	4,093 ^b	3,752						
	SCFPb-2X	4,075 ^b	57,956 ^a	4,025 ^b	54,878 ^a	4,779 ^b	3,007						
	SEM	1,393	13,700	1,689	15,489	1,252							
Fecal LPS, (EU/mL)	Control	7,545 ^b	73,637 ^a	6,325 ^b	107,969 ^a	3,691 ^b	4,883						
	SCFPa	13,203 ^{bB}	73,366 ^a	8,290 ^b	59,951 ^{abA}	8,501 ^b	6,051	0.43	<0.01	-	0.38	-	-
	SCFPb-1X	10,700 ^{ab}	46,644 ^a	6,415 ^b	100,438 ^a	10,287 ^{ab}	6,032						
	SCFPb-2X	11,746 ^b	115,265 ^a	9,819 ^b	100,345 ^a	6,064 ^b	7,405						
	SEM	3,438	18,192	2,424	22,725	2,189							

^{a,b}Means in a row with different superscripts within each Treatment are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts within each Treatment tended to be different ($0.05 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 12 as Post-SARA2.

³Statistical analyses were conducted on log₁₀-transformed data. Presented means are original values prior to transformation.

⁴EU = Endotoxin unit.

Table 3-7. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on rumen and feces free lipopolysaccharide (LPS) concentrations in parity 2 and 3 lactating dairy cows

Item	Parity	Treatment ¹	Stage ²					SEM	P- values ³		
			Pre-SARA1	SARA1	Post-SARA1 (wk7)	SARA2	Post-SARA2 (wk12)		Treatment	Stage	Treat × Stage
Rumen LPS, (EU/mL) ⁴	2	Control	2,945 ^b	99,151 ^a	5,557 ^b	95,940 ^a	2,413 ^b	3,472	0.002	<0.001	0.13
		SCFPa	5,720 ^b	39,819 ^a	6,648 ^b	55,233 ^a	2,950 ^b	3,099			
		SCFPb-1X	5,184 ^b	53,468 ^a	7,266 ^b	74,062 ^a	2,732 ^b	3,530			
		SCFPb-2X	1,831 ^b	49,034 ^a	1,756 ^b	44,381 ^a	2,000 ^b	1,811			
		SEM	1,271	17,255	1,633	19,653	875				
	3+	Control	7.227 ^{bcB}	55,144 ^a	5,966 ^c	48,517 ^{abA}	5,650 ^c	2,678	0.93	<0.001	0.91
		SCFPa	2,876 ^c	76,594 ^{abA}	6,988 ^c	83,695 ^a	8,066 ^{bcB}	3,294			
		SCFPb-1X	5,571 ^b	50,072 ^a	5,915 ^b	74,079 ^a	4,530 ^b	2,732			
		SCFPb-2X	6,897	49,396	7,857	50,176	8,871	3,388			
		SEM	1,209	10,169	1,509	11,647	1,528				

^{a-c}Means in a row with different superscripts within each Treatment are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts within each Treatment tended to be different ($0.05 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 12 as Post-SARA2.

³Statistical analyses were conducted on log₁₀-transformed data. Presented means are original values prior to transformation. In parity 2 cows, the effect of Treatment was significant on average across all Stages. However, no significant difference was observed among Treatments within any given time point.

⁴EU = Endotoxin unit.

Table 3-8. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1.1, and SARA1.2) as well as parity on lipoteichoic acid (LTA) concentration in blood plasma of lactating dairy cows

Item	Treatment ¹	Stage ²			Parity		SEM	P- values ³					
		Pre-SARA1	SARA1.1	SARA1.2	2	3+		Treatment	Stage	Parity	Treat× stage	Treat× parity	Stage× parity
LTA ⁴ , (ng/mL)	Control	0.69	0.76	0.74	0.76	0.70	0.03	0.006	0.07	0.03	0.95	0.69	0.57
	SCFPa	0.68	0.74	0.69	0.75	0.66	0.03						
	SCFPb-1X	0.58	0.64	0.67	0.66	0.61	0.03						
	SCFPb-2X	0.62	0.67	0.66	0.71	0.60	0.03						

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: The first SARA was induced during weeks 5 post-calving. Week 4 was considered as Pre-SARA1, day 2 on week 5 as SARA1.1, and day 5 on week 5 as SARA1.2.

³Statistical analyses were conducted on original values without any transformation. The effect of Treatment was significant on average across all Stages.

Similarly, there was a tendency for the effect of Stage on average across all Treatments. However, no significant difference was observed among Treatments within any given time point.

⁴LTA= Lipoteichoic acid.

Table 3-9. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) and parity on blood acute phase proteins and cytokines of lactating dairy cows

Item ⁴	Treatment ¹	Stage ²					SEM	P- values ³					
		Pre-SARA1	SARA1	Post-SARA1 (wk7)	SARA2	Post-SARA2 (wk10)		Treatment	Stage	Parity	Treat× stage	Treat× parity	Stage× parity
SAA, (µg/mL)	Control	26.66	82.01	35.36	62.25	20.78	6.59	0.05	<0.001	-	0.59	-	-
	SCFPa	16.88 ^{bcB}	83.39 ^a	16.40 ^c	75.08 ^{abA}	22.22 ^{abc}	5.52						
	SCFPb-1X	32.80 ^{AB}	54.25 ^A	18.36 ^{AB}	42.70 ^{AB}	11.42 ^B	4.82						
	SCFPb-2X	10.33 ^b	68.80 ^a	19.40 ^{ab}	48.19 ^{abA}	9.99 ^{bB}	3.99						
	SEM	3.86	11.13	4.07	8.80	2.99							
Hp, (µg/mL)	Control	84.99	95.57	143.55 ^x	77.16 ^y	63.96	19.38	0.04	0.11	-	0.49	-	-
	SCFPa	97.20	155.14	72.51 ^{xy}	230.23 ^x	227.24	33.53						
	SCFPb-1X	136.31	188.90	62.33 ^{xy}	139.28 ^{xy}	101.20	27.10						
	SCFPb-2X	39.38	110.22	33.99 ^y	100.27 ^{xy}	68.70	15.44						
	SEM	21.71	27.48	17.84	26.74	26.63							
LBP, (µg/mL)	Control	10.86	12.29	12.66	12.00	10.69	1.20	0.05	< 0.001	0.19	0.59	0.001	0.22
	SCFPa	8.37	11.57	8.04	9.54	8.99	0.97						
	SCFPb-1X	14.22	13.52	10.56	11.11	7.23	1.19						
	SCFPb-2X	12.01	13.82	9.33	11.29	9.42	1.17						
	SEM	1.28	1.29	1.13	1.11	1.02							
IL-1β, (pg/ml)	Control	10.77	9.44	11.72 ^x	12.45 ^{xy}	16.35	3.43	< 0.001	0.04	< 0.001	0.71	< 0.001	0.79
	SCFPa	3.65	3.90	4.24 ^{xy}	3.74 ^z	5.32	1.21						
	SCFPb-1X	3.82	9.50	10.53 ^{xy}	15.30 ^x	12.91	2.77						
	SCFPb-2X	2.76	3.93	2.57 ^y	4.90 ^{yz}	5.13	1.10						
	SEM	1.43	1.73	1.79	2.19	2.58							
IL-6, (ng/ml)	Control	319.16	339.41	365.48	353.33	319.42	83.37	0.01	0.83	< 0.001	0.99	0.02	0.99
	SCFPa	207.41	288.88	261.91	278.41	253.51	64.12						
	SCFPb-1X	338.42	348.70	437.25	485.75	518.43	110.00						
	SCFPb-2X	383.56	480.39	416.84	491.08	449.98	111.10						
	SEM	83.99	87.53	93.76	95.46	96.55							

^{a-c}Means in a row with different superscripts within each Treatment are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts within each Treatment tended to be different ($0.05 \leq P < 0.1$).

^{x-z}Means in a column with different superscripts within each stage are different ($P < 0.05$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on natural log transformed data. Presented means are original values prior to transformation. For SAA, LBP, there was a tendency for the effect of Treatment on average across all Stages. Similarly, for IL-6, the effect of Treatment was significant on average across all Stages. However, no significant difference was observed among Treatments within any given time point for the indicated variables.

⁴SAA = Serum amyloid A; Hp = haptoglobin; LBP = Lipopolysaccharide (LPS) binding protein; IL-1 β = Interleukin-1 β ; IL-6 = Interleukin-6

Table 3-10. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on lipopolysaccharide (LPS) binding protein (LBP) in different parities.

Item	Parity	Treatment ¹	Stage ²					SEM	P- values ³		
			Pre-SARA1	SARA1	Post-SARA1 (wk7)	SARA2	Post-SARA2 (wk10)		Treatment	Stage	Treat $\times$ Stage
LBP (μ g/mL)	2	Control	9.69	12.37	12.03	12.73	9.74	1.23	0.11	0.006	0.90
		SCFPa	7.67	12.54	8.01	10.13	7.62	0.92			
		SCFPb-1X	9.20	10.34	8.69	9.75	6.30	0.98			
		SCFPb-2X	10.55	12.29	6.99	8.50	5.29	0.94			
		SEM	1.18	1.19	1.07	1.02	0.89				
	3+	Control	12.52	12.23	13.35	11.32	11.74	2.14	0.001	0.29	0.19
		SCFPa	8.93	10.16	7.80	8.70	10.62	1.66			
		SCFPb-1X	23.41	18.62	12.38	12.28	8.60	2.58			
		SCFPb-2X	14.18	15.55	12.79	15.70	18.18	2.73			
		SEM	2.58	2.41	2.12	2.07	2.21				

¹Treat: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on natural log transformed data. Presented means are original values prior to transformation. In parity 2 cows, the effect of Stage was significant on average across all Treatments. However, no significant difference was observed among Stages within any given Treatment for the indicated variables. In parity 3+ cows, the effect of treatment was on average across all stages. However, no significant difference was observed among Treatments within any given time point.

Table 3-11. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on interleukin-1 β (IL-1 β) in different parities.

Item	Parity	Treatment ¹	Stage ²					SEM	P- values ³		
			Pre-SARA1	SARA1	Post-SARA1 (wk7)	SARA2	Post-SARA2 (wk10)		Treatment	Stage	Treat $\times$ Stage
IL-1 β (pg/ml)	2	Control	15.74	15.91 ^{xy}	22.01	17.32 ^{xy}	21.25 ^{XY}	6.57	<0.001	0.48	0.78
		SCFPa	4.44	5.09 ^y	5.50	4.06 ^y	4.95 ^Y	1.65			
		SCFPb-1X	10.65	21.62 ^x	36.34	49.07 ^x	38.83 ^x	9.91			
		SCFPb-2X	13.76	17.40 ^{xy}	10.76	19.61 ^x	17.30 ^{XY}	5.49			
		SEM	3.75	4.60	5.39	5.60	5.95				
	3+	Control	10.58 ^x	8.23 ^x	9.16 ^x	12.73 ^x	17.98 ^x	4.84	<0.001	0.29	0.19
		SCFPa	-	3.97 ^{xy}	4.69 ^{xy}	5.05 ^{xy}	8.59 ^{XY}	1.66			
		SCFPb-1X	2.09 ^{xy}	5.98 ^x	4.27 ^{xy}	6.52 ^{xy}	5.76 ^{XY}	2.00			
		SCFPb-2X	0.58 ^y	1.20 ^y	0.98 ^y	1.77 ^y	2.32 ^Y	0.54			
		SEM	1.27	1.68	1.60	2.24	2.96				

^{x,y}Means in a column with different superscripts within each stage are different ($P < 0.05$).

^{x,Y}Means in a column with different superscripts within each stage tended to be different ($0.05 \leq P < 0.1$).

¹Treat: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on natural log transformed data. Presented means are original values prior to transformation.

Table 3-12. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X) and stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on interleukin-6 (IL-6) in different parities.

Item	Parity	Treatment ¹	Stage ²					SEM	P- values ³		
			Pre-SARA1	SARA1	Post-SARA1 (wk7)	SARA2	Post-SARA2 (wk10)		Treatment	Stage	Treat × Stage
IL-6 (ng/ml)	2	Control	397.07	412.73	439.22	442.13	409.57	156.12	0.01	0.83	0.99
		SCFPa	253.84	352.48	315.58	352.62	243.59	109.49			
		SCFPb-1X	626.16	636.51	954.80	876.82	1103.45	305.83			
		SCFPb-2X	555.30	849.54	747.92	864.20	705.71	274.69			
		SEM	165.18	193.69	212.77	214.42	201.55				
	3+	Control	389.59	418.68	456.28	423.60	373.75	107.92	0.50	0.93	0.99
		SCFPa	217.67	321.98	305.97	294.33	459.25	86.49			
		SCFPb-1X	-	288.13	262.54	420.02	323.47	124.75			
		SCFPb-2X	409.44	411.91	355.14	425.77	443.81	112.31			
		SEM	135.74	93.95	97.28	101.17	116.02				

¹Treat: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126 g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on natural log transformed data. Presented means are original values prior to transformation. In parity 2 cows, the effect of Treatment was significant across all Stages. However, no significant difference was observed among Treatment within any given time point.

CHAPTER FOUR

MANUSCRIPT 2

Postbiotics from *Saccharomyces cerevisiae* fermentation stabilize microbiota in rumen liquid digesta during grain-based subacute ruminal acidosis (SARA) in lactating dairy cows

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4.1 Abstract

Subacute ruminal acidosis (SARA) is a common metabolic disorder of high yielding dairy cows, during which the rumen pH is reversibly reduced several hours each day. It is associated with dysbiosis of the gut microbiome and host inflammation. This study evaluated the impact of two postbiotics, i.e. *Saccharomyces cerevisiae* fermentation products on microbiota in rumen liquid of lactating dairy cows subjected to repeated grain-based SARA challenges. A total of 32 rumen cannulated cows were randomly assigned to four treatments from 4 wk before until 12 wk after parturition. Treatment groups included a Control diet or diets supplemented with postbiotics (SCFPa, 14 g/d Original XPC; SCFPb-1X, 19 g/d NutriTek; SCFPb-2X, 38 g/d NutriTek Diamond V, Cedar Rapids IA). Grain-based SARA challenges were conducted during wk 5 (SARA1) and wk 8 (SARA2) after parturition by replacing 20% DM of the base total mixed ration

(TMR) with pellets containing 50% ground barley and 50% ground wheat. Total DNA from rumen liquid digesta samples was subjected to V3-V4 16S rRNA gene amplicon sequencing. Characteristics of rumen microbiota were compared among treatments and stages. Our results showed that both SARA challenges reduced the richness and diversity and changed the β -diversity of microbiota in rumen liquid digesta, and altered its taxonomic composition and predicted functionality, including carbohydrates and amino acids metabolic pathways. The challenges reduced the number of significant associations among different taxa, number of hub taxa and their composition in the microbial co-occurrence networks. Supplementation with postbiotics, and especially SCFPb-2X, enhanced the robustness of the rumen microbiota. Supplemented cows in these groups had less fluctuation in relative abundances of community members when exposed to SARA challenges. Postbiotic supplementation promoted the populations of lactate utilizing and fibrolytic bacteria, including members of Ruminococcaceae and Lachnospiraceae, and also increased the number of positive connections and the hub taxa during non-SARA and SARA stages. supplementation with SCFPb-2X prevented the bacterial fluctuations in the abundances of hub taxa that were positively correlated with the acetate concentration, α - and β -diversity metrics in rumen liquid digesta. In conclusion, induction of SARA challenges disrupted rumen liquid microbiota of lactating dairy cows and supplementation with SCFP postbiotics could attenuate adverse effects of SARA on rumen liquid associated microbiota.

Key words: Postbiotics; *Saccharomyces cerevisiae* fermentation products (SCFP); SARA; rumen microbiota

4.2 Introduction

Excessive grain feeding in cattle is associated with gut health disorders, such as acute lactic ruminal acidosis and subacute ruminal acidosis (SARA) (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2018). These disorders can occur when animals are rapidly transitioned from low grain-high forage diets to high grain-low forage diets, as rumen pH reduces due to increased dietary starch digestion and reduced rumen buffering. The etiology of SARA is complex and multifactorial (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2018), as it does not result in an accumulation of lactate, but increased concentrations of volatile fatty acids (VFAs) that reduce the rumen pH between 5.2 and 5.6 for at least 3 hours per day (Gozho et al., 2005). Apart from dietary factors, such as dietary energy, fiber and starch contents, animal characteristics such as robustness of their rumen and hindgut microbiomes, feeding behavior and capacity of rumen epithelium for absorption of VFA and lactate affects the severity of SARA (Guan et al., 2008; Beauchemin and Penner, 2009; Khafipour et al., 2009b; Penner et al., 2009; Huws et al., 2018). Robustness is the capacity of a microbial community to resist changes caused by the stressors such as dietary changes, and the degree of robustness of the rumen and hindgut microbiomes is a key determinant of the microbiome-linked pathology (Khafipour et al., 2016). In the presence of excess starch content of the diet, insufficient dietary coarse fiber, and a reduced pH, a less robust rumen microbiome rapidly loses its evenness and becomes dominated by a smaller number of taxa (Russell et al., 1981; Khafipour et al., 2016). Low rumen pH may cause the decrease of the relative abundance of fibrolytic bacteria and the increase of amylolytic bacteria in the rumen (Mao et al., 2013; Petri et al., 2013). This can result in rapid accumulation of organic acids such as VFAs that reduce the rumen pH, and the reduction in the digestive tract

barrier function. Consequently, increased concentrations of immunogenic compounds in the digestive tract such as lipopolysaccharides (LPS) and histidine, and the translocation of these and other toxic compounds from the digestive tract into the systemic circulation can result in inflammatory responses (Emmanuel et al., 2007; Khafipour et al., 2009b; Ghaffari et al., 2017). Improved ration formulation, e.g., via introduction of effective neutral detergent fiber (eNDF) concept, and management practices, e.g., via total mixed ration (TMR), and feed bunk management, have reduced the occurrence of acute lactic ruminal acidosis (Pritchard and Bruns, 2003; Nagaraja and Lechtenberg, 2007; Beauchemin and Penner, 2009). However, SARA is still highly prevalent and commonly goes unnoticed resulting in reduced animal production and efficiency of production, compromised animal health and development of secondary diseases that lead to high herd culling rates (Plaizier et al., 2012b).

One strategy for the prevention of SARA is supplementation with compounds that improve the robustness of digestive tract's microbiome. These compounds can be divided into two categories, including those with suppressive effects on other microorganisms, such as antibiotics and ionophores, and those with promotive effects, such as probiotics and postbiotics (Khafipour, 2021). Suppressive supplements improve the robustness of microbiomes by the suppression of fast-growing gram-positive bacteria, which allows slower growing fibrolytic organisms to maintain their populations, which in turn prevents from loss of evenness and richness (Mao et al., 2013; Khafipour, 2021). However, the suppressive approach is accompanied by side effects, such as the development of a resistome against suppressor compounds, and reductions in the diversity of carbohydrate active enzymes (CAZyme) in the microbial community (Khafipour, 2021). In contrast,

the use of promotive supplements, such as the use of postbiotics, improves the robustness of microbiome by the provision of metabolites that feed a wide range of bacteria, including those with fibrolytic activity, with a competitive edge against faster grower species that allows them to maintain their populations within the microbial community (Zhu et al., 2017; Tun et al., 2020). As such, a postbiotic approach can promote the robustness of a microbiome without affecting its resistome and reducing its CAZyme capability (Khafipour, 2021).

A Postbiotic *Saccharomyces cerevisiae* fermentation product (SCFP) (Original XPC, Diamond V, Cedar Rapids, IA) was effective in enhancing the robustness of gastrointestinal microbiome in dairy cows subjected to a grain-based SARA challenge (Tun et al., 2020). In our companion manuscripts (Khalouei et al., 2020; Guo et al., 2022), we showed that a next generation SCFP (NutriTek, Diamond V) was more effective in stabilizing the rumen environment and reducing pro-inflammatory status during SARA compared to XPC. Therefore, we hypothesized that: 1) Grain-based SARA changes composition and reduces the predicted functionality of the microbiome in rumen liquid digesta, and this effect is more severe in a second SARA challenge (SARA 2) compared to the first challenge (SARA 1); 2) SCFPs (XPC and NutriTek) reduce the adverse effects on the composition and functionality of the microbiome in rumen liquid digesta with Nutritek being more effective with a dose dependency. The objectives of this study were to test these hypotheses by comparing the effects of two commercially available SCFP (XPC, Diamind V, Cedar Rapids, IA and NutriTek) on characteristics of rumen liquid microbiota in Holstein dairy cows subjected to two grain-induced SARA challenges during early to mid-lactation.

4.3 Materials and Methods

The protocol used in this study was approved by the University of Manitoba Animal Care Committee (Protocol # F14-038) and followed the guidelines of the Canadian Council for Animal Care (CCAC, 1993).

4.3.1 Animals, Diet and Experimental design

As described previously (Guo et al., 2022), a total of 32 cannulated lactating dairy cows were used in a randomized complete block design with 8 blocks. Cows were blocked based on parity, previous milk yield, and calving date, and were rumen cannulated approximately 12 wk before their expected parturition. Cows were fully recovered from the surgery before the start of the study, as evaluated by farm veterinarians. Within each block, cows were randomly assigned to one of four treatments that were applied from 4 weeks before until 12 weeks after parturition and included: 1) no SCFP (Control), 2) 14 g/d Original XPC (SCFPa, Diamond V), 3) 19 g/d NutriTek (SCFPb-1X, Diamond V), and 4) 38 g/d NutriTek (SCFPb-2X, Diamond V).

Supplements were mixed with 126, 121, or 102 g/d of ground corn for treatments 2 to 4, respectively, and fed once daily as a top-dress. Cows were fed a prepartum diet containing 38.7% DM neutral detergent fiber (NDF), 15.5% DM crude protein (CP) and 17.6% DM starch from 4 weeks before parturition and were switched to a lactation diet with 34.9% DM NDF, 17.9% DM CP and 18.6% DM starch until 12 weeks after parturition, except for SARA challenge weeks. During wk 5 and wk 8 post- parturition, two grain-based SARA challenges were induced (SARA1, SARA2) by replacing 20% of

the DM of the lactation TMR with pellets containing 50% ground barley and 50% ground wheat, resulting in a SARA induction diet containing 28.2% DM NDF, 17.2% DM CP and 27.9% DM starch. This replacement was completed gradually over 3 days before the start of the SARA1 and SARA2 challenges. Each SARA challenge lasted for 7 days after which cows were returned to lactation TMR to washout the effects of SARA for two weeks. Cows were fed TMR *ad libitum* once daily at 0900 h, allowing for between 5 and 10% feed refusals. Animals had free access to fresh water during the whole experiment. Detailed description of feed sample collection and analyses, chemical and nutrient composition of diets, and confirmation of successful SARA induction (rumen pH reduction) were reported previously (Khalouei et al., 2020; Guo et al., 2022). Cows were housed in individual stalls and had free access to fresh water during the whole experiment.

Sample Collection. Whole rumen content samples were collected from 5 sites (cranial, caudal, dorsal, caudal ventral, and caudal dorsal) of the rumen through the rumen canula at 6 h after feed delivery, as described in Chapter 3. Rumen solid and liquid digesta were separated with a Bodum coffee filter plunger (ODUM ® AG, Baar, Switzerland) as described in the companion manuscript (Guo et al., 2022). One mL of rumen liquid was subsampled, snap frozen in liquid nitrogen and stored at -80°C for further microbiota analysis.

DNA Extraction. Frozen rumen liquid samples were thawed on ice and centrifuged at $15,000 \times g$ for 10 min to collect the pellets. Their DNA was extracted from the sediment using *Quick-DNA*TM ZR Fecal/Soil DNA kits (D6010; Zymo Research Corp., Orange, CA) following manufacturer's procedures. These included a 2 min bead-beating step at

1750 strokes per minute using Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ, USA) for the mechanical disruption of bacterial cells. Obtained DNA was stored at -80°C in aliquots of 100 ng/μL (stock) of elution buffer. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and samples were normalized to 20 ng/μL for following MiSeq Illumina short-read sequencing.

PCR Amplification and Construction of Sequencing Libraries. The PCR was targeted to amplify V3-V4 hypervariable regions of the bacterial 16S rRNA genes using modified F338/R806 primers (Derakhshani et al., 2016). Briefly, the forward PCR primer was indexed with 12-base Golay barcodes, allowing for multiplexing of samples. For each sample, PCR reaction was performed in duplicate and contained 3.0 μL of extracted genomic DNA, 1.0 μL of each forward and reverse primer (5 μM), 0.5 μL of 20 mg/mL BSA (Thermo Fisher Scientific), 7.0 μL nuclease-free water (Thermo Fisher Scientific), and 12.5 μL of 5 Prime Hot MasterMix (5 Prime Inc., Gaithersburg, MD, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 32 amplification cycles at 94°C for 30 s, 55°C for 20 s, and 72°C for 20 s, with a final extension step at 72°C for 5 min in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). Subsequently, the sequencing library was generated and sequenced using a MiSeq Reagent Kit V3 (600-cycle; Illumina, San Diego, CA, USA) as described by Derakhshani et al. (Derakhshani et al., 2016) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada.

4.3.2 Statistical Analysis

Bioinformatics Analyses of Microbiota Data. The QIIME2 2023.2. (Bolyen et al., 2019) pipeline was used to analyze the 16S rRNA gene amplicon sequencing data. The DADA2 algorithm was used for quality control and the feature table was constructed where sequences were assigned into amplicon sequence variants (ASVs). Subsequently, taxonomy was classified and aligned against the Silva database (v.128). Phylogenetic trees were built for further comparison among microbial communities.

Prior to performing downstream analyses, the resulting feature table was filtered to remove samples with low sequencing depths (< 6072 sequences per sample). Subsequently, community α -diversity [Chao1, Shannon's diversity index, Observed Features index, Faith's Phylogenetic Diversity (PD) index and Pielou's Evenness index] and β -diversity (Jaccard distance, Bray-Curtis distance, unweighted UniFrac distance, weighted UniFrac distance) metrics were conducted using QIIME2 default scripts at an even depth per sample. Non-metric multidimensional scaling (nMDS) was applied on the resulting Bray-Curtis distance matrices to generate two-dimensional plots using default settings of the PRIMER-E software (v.7.0.17) (Clarke and Gorley, 2015).

Statistical Analyses of Microbiota Data. For comparison of α -diversity and the relative abundances of bacterial taxa, a parametric approach was implemented. Firstly, the normality of distributions of residuals was tested using UNIVARIATE procedure of SAS (v 9.4, SAS Institute Inc., Cary, NC, USA) by Shapiro-Wilk's statistic. If the residuals were not normal, then the data were raised to the power of lambda to achieve normality. The lambda was calculated by Box-Cox transformation using TRANSREG

procedure of SAS (v.9.4). Original and transformed data were then analyzed by the MIXED procedure of SAS using the model:

$$Y_{ijk} = \mu + T_i + S_j + P_k + (T \times S)_{ij} + (T \times P)_{ik} + (P \times S)_{kj} + (T \times P \times S)_{ijk} + e_{ijk}$$

The variable Y_{ijk} was dependent on μ as the average experimental value and fixed effects of treatment T_i (i =Control, SCFPa, SCFPb-1X and SCFPb-2X), stage S_j [j =wk (-4), wk(-1), wk1, ..., wk12], parity P_k (k =2 or 3+ parity), and interactions $(T \times S)_{ij}$, $(T \times P)_{ik}$, $(P \times S)_{kj}$ and $(T \times S \times P)_{ijk}$. Block was considered a random factor, and stage as repeated measure. The PDIFF option was applied for pairwise comparisons among treatments, parities, stages and their interactions. Comparisons of least square means were conducted using Tukey HSD tests. Contrast comparisons were made between control and SCFP, SCFPa and SCFPb, and SCFPb-1X and SCFPb-2X. If the effect of parity had a P-value more than 0.1, parity and its interactions were removed from the model. Results were presented as least square means with their pooled standard errors (SEM). For interpretation of results, the data that was transformed to achieve normality were presented as means from the original data within tables. Significant effects were considered at $P < 0.05$ and tendencies were discussed at $0.05 \leq P < 0.1$.

Permutational multivariate analysis of variance (PERMANOVA; implemented in PRIMER-E software (v.7.0.17)) was used for the comparison of β -diversity metrics (Clarke and Gorley, 2015). Label permutations ($n = 9999$) were used in PERMANOVA to estimate the distribution of test statistics under the null hypothesis that within-group UniFrac or Bray-Curtis measures were not significantly different from between-group measures. The fixed and random factors were incorporated into a PERMANOVA model as described for α -diversity. To support the results of PERMANOVA, PERMDISP

analysis in PRIMER-E was used to detect the effect of the dispersions on β -diversity of the rumen liquid microbiota (Clarke and Gorley, 2015).

Compositional dynamics of rumen liquid bacterial communities were assessed using the Metagenomic Longitudinal Differential Abundance (MetaLonDA) method with the edgeR package in R (Metwally et al., 2018). To achieve this, taxonomic profiles for all samples from all cows were integrated into one count table and normalized with cumulative sum scaling (CSS). Pairwise comparisons were made between Control vs. SCFPa, Control vs. SCFPb-1X, and Control vs. SCFPb-2X. Within each comparison, the longitudinal profiles were fitted with a negative binomial smoothing spline. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate (FDR) estimation (Benjamini and Hochberg, 1995). Data were presented both at the phylum and genus levels.

Functional Prediction of Rumen Liquid Microbiota. Predicted functions including amino acid, lipid and carbohydrates metabolic pathways of rumen liquid microbiota were assessed by rumen-specific Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt; CowPI) (Wilkinson et al., 2018) after filtering the feature table with the average ASV count $> 0.005\%$. The differences between Control and SCFP supplemented groups were analyzed by STAMP (v.2.1.3) using ANOVA test (Parks et al., 2014). Significant differences were adjusted by Tukey-Kramer test and then were identified when $P < 0.05$ after multiple testing corrections by Benjamini-Hochberg's FDR correction (Benjamini and Hochberg, 1995).

Co-occurrence Analysis. A correlation network analysis (CoNet) was used to determine microbial association network and the general organization structure of the

microbial community (Faust and Raes, 2016). Positive /negative connections indicates co-occurrence/mutual-exclusion relationships among taxa respectively, and hub taxa were identified when they had more than 15 positive/negative connections with other community members (Faust and Raes, 2016) as described previously (Derakhshani et al., 2018; Zhang et al., 2018). The degree of connectedness, a measure used to determine the influential capacity of bacterial taxa (Trosvik and de Muinck, 2015), was explored at the phylum level by dividing the total number of positive and negative edges observed for each phylum by its relative abundance in the community. Hub ASVs were picked when they had > 15 edges (connections) with other members in the community.

Spearman's rank correlation coefficient (ρ) was used to explore the relationships between hub ASVs and biodiversity (α - and β -diversity metrics), rumen VFAs as well as rumen ammonia concentrations. Resulting correlation matrices were visualized in heatmaps generated by the Corrplot package of R (Wei et al., 2017).

4.4 Results

In this study, we evaluated the effects of these SCFPs (SCFPa and SCFPb) and grain-based SARA challenges on characteristics of microbiota in rumen liquid digesta.

4.4.1 Alpha- and beta-diversity dynamics of rumen liquid microbiota

There were 26,532 ASVs found in samples and an average of $23,720 \pm 6,492$ reads per sample through 16S rRNA gene sequencing. The richness and diversity of the rumen liquid microbiota were reduced ($P < 0.001$) by both SARA challenges across treatment groups (Table 4-1). There were no differences in these alpha-diversity indices between

the two SARA challenges. Within each experimental stage, evenness, but not richness, was affected by treatments. During the cows on the SARA1/1 stage, SCFPb-2X treatment maintained greater microbial Pielou's evenness ($P = 0.01$) compared to cows on the SCFPa and SCFPb-1X treatments, and tended ($P = 0.08$) to have greater evenness of microbiota in liquid rumen digesta compared to the Control cows. The evenness in SCFPb-1X cows was lower than in cows on other SCFP treatments during the post-SARA1 and post-SARA2 stages (wk 10; $P < 0.05$). During the SARA2/1 stage, the evenness in SCFP-2X cows was greater ($P = 0.02$) than that in SCFPa cows, but not different from those in SCFPb-1X and Control cows. Results from PERMANOVA showed that both treatment ($P < 0.01$) and stage ($P < 0.01$) affected the β -diversity of rumen liquid microbiota (Figure 4-1). Moreover, the treatment effect assessed by PERMDISP was significant ($P = 0.02$). The stage effect was also significant on β -diversity of the microbiota in rumen liquid digesta in all of the treatment groups according to the PERMDISP analyses ($P > 0.05$, Figure 4-1).

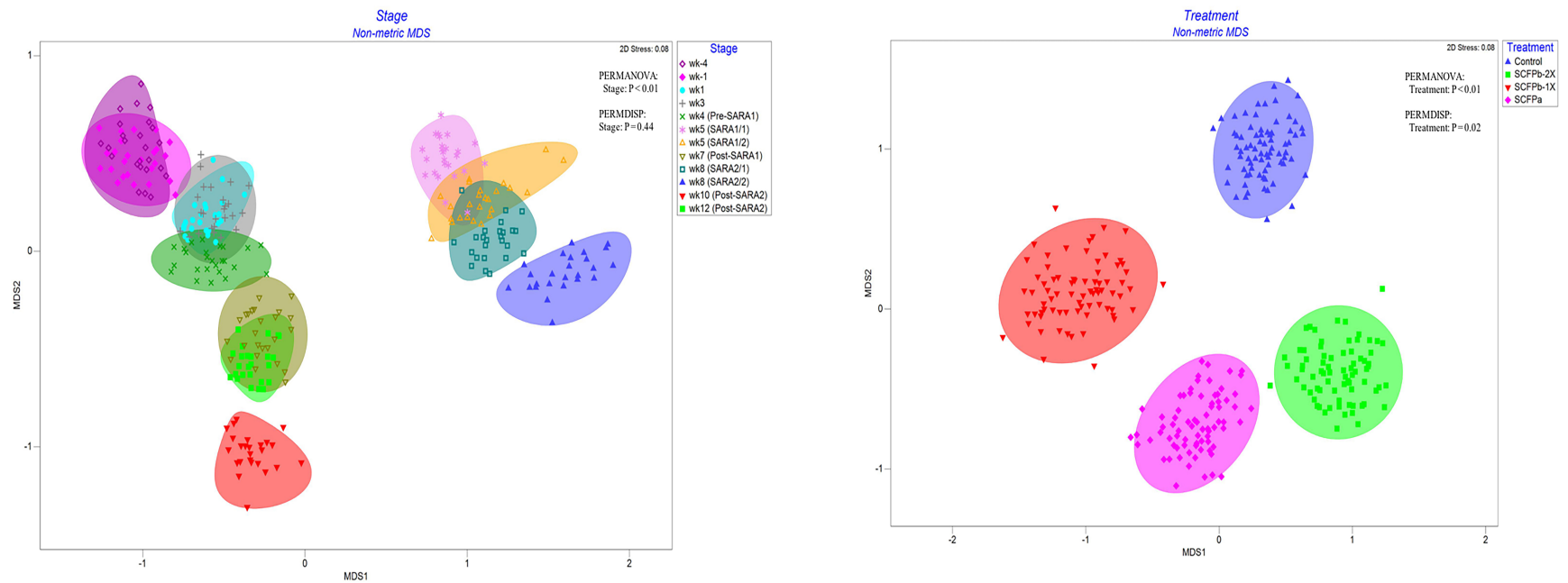


Figure 4-1. nMDS of Bray-Curtis distances of rumen liquid microbial communities. The ASV table was normalized using cumulative sum scaling (CSS) transformation. Permutational multivariate analysis of variance (PERMANOVA) was used to detect the distinction of clustering patterns between treatments and stages. $P < 0.05$ was considered as significant different. The effect of block was considered as random factor in all comparisons. The experimental stage was started from 4 weeks before until 12 weeks after calving. SARA challenges were conducted on wk 5 and wk 8 after parturition. Rumen samples were taken weekly but twice during SARA weeks (SARA1/1, SARA1/2, SARA2/1, SARA2/2). Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 and 12 as Post-SARA2.

4.4.2 Compositional dynamics of rumen liquid microbiota in response to SARA challenges

In total, 17 phyla and 471 genera were identified. The microbiota in rumen liquid digesta was dominated by members of phylum Firmicutes (48~52%) and Bacteroidetes (37~42%), and of Proteobacteria (3.8~4.9%), Actinobacteria (0.3~0.5%), Tenericutes (0.2~0.4%), and Fibrobacteres (0.1~0.3%) were observed. The average relative abundances of the major bacterial phyla in rumen liquid microbiota during non-SARA (Pre-SARA1, Post-SARA1 and Post-SARA2) and SARA (SARA1, SARA2) stages in each treatment group were summarized in Table 4-2. Both Grain-based SARA challenges reduced the relative abundance of Actinobacteria (0.56 vs. 0.32%, $P < 0.05$), Fibrobacteres (0.38 vs. 0.15%, $P < 0.05$) and Tenericutes (0.64 vs. 0.10%, $P < 0.05$), while increased that of Proteobacteria (2.98 vs. 6.67%, $P < 0.01$). The second SARA challenge reduced the relative abundance of Firmicutes compared with post-SARA2 (47.13 vs. 53.51%, $P = 0.04$), but not with other stages ($P > 0.05$). However, SARA challenges did not influence the relative abundance of Bacteroidetes in rumen liquid microbiota. No treatment effect was observed on the relative abundances of these phyla except for Fibrobacteres, which relative abundance in the SCFPb-1X treatment tended to be lower than in the SCFPb-2X ($P = 0.07$) treatment. The ratio of Firmicutes: Bacteroidetes was not affected by SARA or SCFP supplementation ($P > 0.1$). Comparisons of compositional shifts in Firmicutes and Bacteroidetes populations among SCFP treatments groups are given in Figure 4-2.

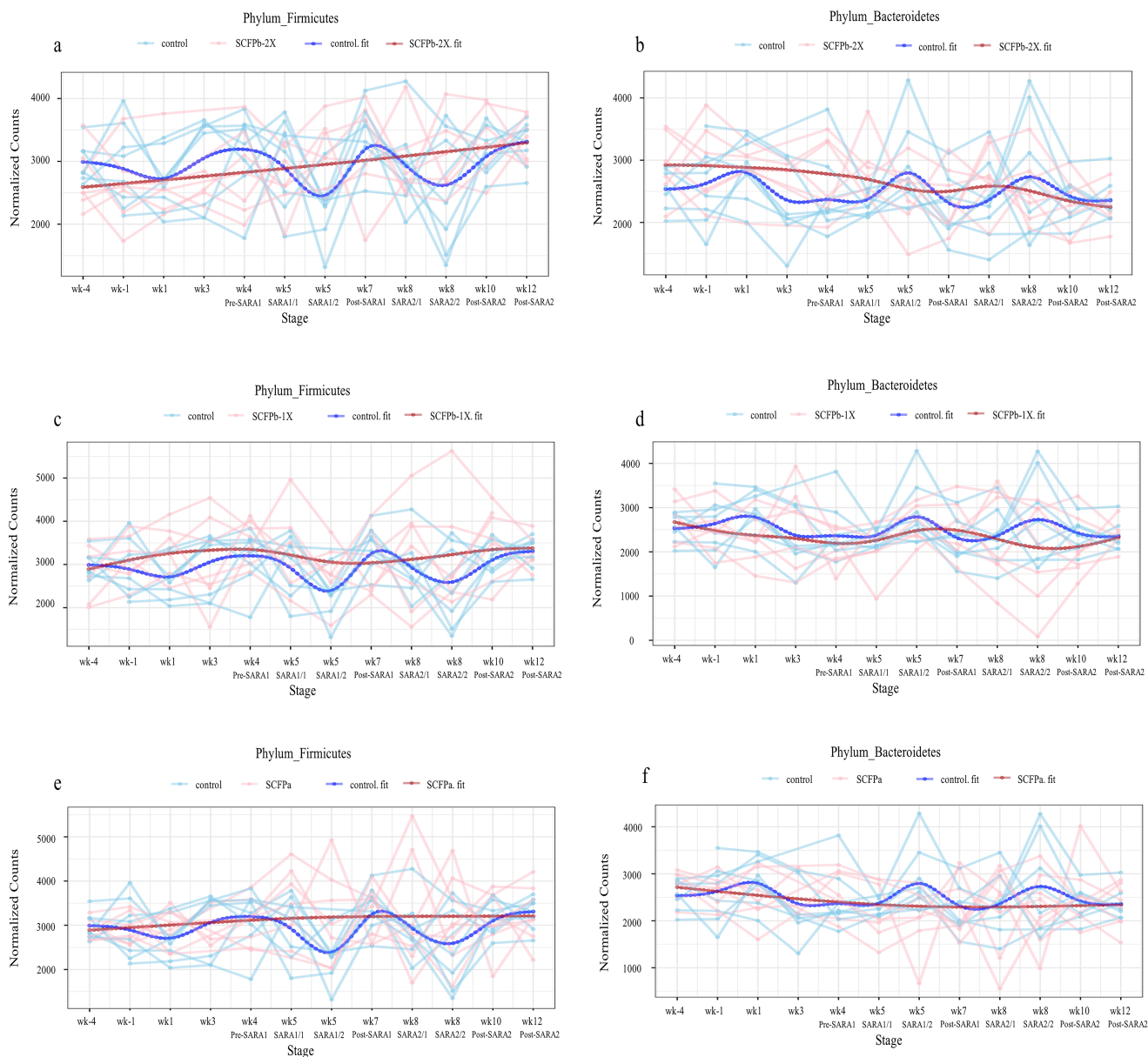


Figure 4-2. Longitudinal shifts in Firmicutes and Bacteroidetes proportions in rumen liquid microbiota. Metagenomic Longitudinal Differential Abundance (MetaLonDA) was used to test the longitudinal changes in rumen liquid microbiota at the phylum level as lactation progressed. The ASV table was normalized using cumulative sum scaling (CSS) transformation. The longitudinal profiles in each group were fitted with a negative binomial smoothing spline. Blue color represents control and red represents SCFP groups. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. a, b) Longitudinal shifts in Firmicutes and Bacteroidetes

proportions, respectively, in control vs. SCFPb-2X group. c, d) Longitudinal shifts in Firmicutes and Bacteroidetes proportions, respectively, in control vs. SCFPb-1X group. e, f) Longitudinal shifts in Firmicutes and Bacteroidetes proportion, respectively, in control vs. SCFPa group. Cows received treatments from 4 weeks before until 12 weeks after parturition. SARA challenges were conducted on wk 5 and wk 8. Rumen samples were collected weekly but twice during SARA weeks (SARA1/1, SARA1/2, SARA2/1, SARA2/2).

Higher fluctuations in the relative abundances of Firmicutes and Bacteroidetes during the first week after parturition and during both SARA challenge stages were observed in the Control cows compared to SCFP supplemented cows.

At the genus level, taxa that were significantly different in relative abundance among SARA stages and SCFP treatments as determined by the MetaLonDA, are summarized in Figures 4-3 to 4-5. Figure 4-3 shows taxa whose relative abundances were increased by the Control or SCFPb-2X treatments throughout the 16 wk study period. Overall, 83 taxa were increased by the Control or SCFPb-2X treatments during corresponding experimental stage. The SCFPb-2X treatment increased the relative abundances of several taxa, including members of Bacteroidales RF16, Ruminococcaceae, Prevotellaceae, Lachnospiraceae, Rikenellaceae RC9, *Selenomonas* and *Spirochaeta* compared to the Control treatment during the SARA stages ($P < 0.05$). When comparing the Control and SCFPb-1X treatments, the relative abundances of 83 bacterial taxa were increased by one of these two treatment groups within corresponding experimental stage (Figure 4-4).

Table 4-1. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (from 4w before until 12 wk after calving) on alpha-diversity of rumen liquid microbiome in lactating dairy cows

Item	Treatment ¹	Stage ²												SEM	P-value ³		
		wk -4	wk -1	wk 1	wk 3	wk 4	wk 5	wk 5	wk 7	wk 8	wk 8	wk 10	wk 12		Treat	Stage	Int ⁴
						Pre-SARA1	SARA1/1	SARA1/2	Post-SARA1	SARA2/1	SARA2/2	Post-SARA2	Post-SARA2				
Chao1	Control	784.29 ^{ab}	813.14 ^{ab}	775.42 ^{ab}	879.10 ^a	772.38 ^{ab}	520.65 ^{abA}	450.21 ^{bc}	684.25 ^{ab}	448.00 ^c	351.05 ^c	732.26 ^{ab}	713.63 ^a	39.66	0.57	<0.0001	0.12
	SCFPa	778.59 ^a	762.44 ^a	615.37 ^{abc}	752.14 ^{ab}	766.40 ^a	420.12 ^{bc}	376.62 ^c	740.39 ^a	398.75 ^c	413.75 ^{bc}	824.06 ^a	607.77 ^{abc}	39.94			
	SCFPb-1X	786.73 ^{aA}	762.59 ^a	856.86 ^a	734.62 ^a	774.34 ^a	398.01 ^{bB}	464.15 ^{ab}	589.01 ^{ab}	345.01 ^b	332.86 ^b	622.01 ^{ab}	768.37 ^a	40.92			
	SCFPb-2X	664.43	756.15	590.31	704.01	637.30	488.01	509.76	637.18	457.58	496.01	752.52	683.32	41.09			
Shannon's diversity	Control	8.78 ^a	8.77 ^a	8.54 ^a	8.64 ^a	8.53 ^a	7.33 ^b	7.05 ^b	8.52 ^a	6.95 ^b	6.41 ^b	8.56 ^a	8.52 ^a	0.11	0.62	<0.0001	0.03
	SCFPa	8.58 ^a	8.71 ^a	8.08 ^{bc}	8.80 ^a	8.76 ^a	6.52 ^d	6.68 ^d	8.60 ^a	6.32 ^d	6.73 ^d	8.53 ^{ab}	7.92 ^c	0.11			
	SCFPb-1X	8.71 ^{ab}	8.66 ^{ab}	8.82 ^a	8.08 ^{bc}	8.60 ^{ab}	6.55 ^d	7.17 ^{cd}	7.77 ^c	6.50 ^d	6.57 ^d	8.04 ^c	8.65 ^{ab}	0.12			
	SCFPb-2X	8.49 ^a	8.50 ^a	8.29 ^{ab}	8.55 ^a	8.43 ^a	7.71 ^{bc}	7.26 ^c	8.43 ^a	7.27 ^c	7.21 ^c	8.68 ^a	8.65 ^a	0.12			
Pielou's Evenness	Control	0.92 ^a	0.92 ^a	0.90 ^a	0.89 ^a	0.90 ^a	0.82^{bY}	0.81 ^b	0.91 ^{ax}	0.80 ^b	0.77 ^b	0.91 ^{ax}	0.91 ^{aX}	0.01	0.13	<0.0001	0.01
	SCFPa	0.90 ^a	0.92 ^a	0.88 ^a	0.93 ^a	0.92 ^a	0.77^{by}	0.80 ^b	0.91 ^{ax}	0.75 ^{by}	0.80 ^b	0.91 ^{ax}	0.86 ^{ayY}	0.01			
	SCFPb-1X	0.91 ^a	0.91 ^a	0.91 ^a	0.86 ^{bc}	0.90 ^{ab}	0.78^{dy}	0.83 ^{cd}	0.86 ^{cy}	0.79 ^d	0.80 ^d	0.87 ^{bey}	0.91 ^{aX}	0.01			
	SCFPb-2X	0.91 ^a	0.90 ^{ab}	0.91 ^a	0.91 ^a	0.91 ^a	0.88^{bexX}	0.83 ^d	0.91 ^{ax}	0.83 ^{cdx}	0.81 ^d	0.91 ^{ax}	0.92 ^{ax}	0.01			
Faith's Phylogenetic diversity	Control	122.97 ^{ab}	125.06 ^{ab}	116.30 ^{ab}	130.62 ^a	116.76 ^b	76.74 ^c	71.70 ^c	110.09 ^b	71.94 ^c	59.83 ^c	111.43 ^b	111.75 ^b	3.92	0.54	<0.0001	0.08
	SCFPa	117.16 ^a	117.47 ^a	99.66 ^b	121.58 ^a	116.57 ^a	64.05 ^c	62.83 ^c	109.90 ^{ab}	62.82 ^c	66.75 ^c	111.65 ^{ab}	98.69 ^b	3.94			
	SCFPb-1X	120.02 ^{ab}	120.06 ^{ab}	127.16 ^a	114.91 ^{ab}	119.27 ^{ab}	63.53 ^d	72.29 ^d	95.70 ^c	59.28 ^d	59.50 ^d	105.34 ^{bc}	114.64 ^{ab}	4.12			
	SCFPb-2X	109.99 ^a	117.54 ^a	98.69 ^{ac}	113.09 ^a	101.63 ^a	78.00 ^b	77.33 ^b	102.72 ^a	69.48 ^b	79.06 ^{bc}	117.99 ^a	111.23 ^a	4.13			
Observed features	Control	757.39 ^{ab}	768.50 ^{ab}	727.87 ^{ab}	832.10 ^a	732.62 ^{ab}	489.62 ^c	426.76 ^c	658.37 ^b	428.00 ^c	333.13 ^c	691.37 ^{ab}	678.25 ^b	36.60	0.61	<0.0001	0.11
	SCFPa	729.87 ^a	714.01 ^a	587.87 ^a	698.21 ^a	732.12 ^a	398.83 ^b	357.00 ^b	695.62 ^a	367.12 ^b	388.50 ^b	743.25 ^a	579.50 ^a	36.83			
	SCFPb-1X	750.01 ^a	734.44 ^a	817.01 ^a	701.13 ^{ab}	740.72 ^a	382.72 ^d	448.44 ^{cd}	569.29 ^{bc}	334.86 ^d	330.15 ^d	603.15 ^{abc}	728.57 ^a	38.92			
	SCFPb-2X	648.44 ^{ab}	723.58 ^a	584.15 ^{bcd}	685.04 ^a	624.86 ^{ab}	481.86 ^{cd}	486.86 ^{bcd}	621.29 ^{abc}	442.72 ^d	481.15 ^{cd}	730.72 ^a	674.56 ^a	39.04			

^{a-c}Means in a row with different superscripts among Stages are different ($P < 0.05$).

^{x,y}Means in a column with different superscripts among treatments are different ($P < 0.05$).

^{x,y}Means in a column with different superscripts among treatments are tended to be different ($0.5 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during wk 5 (SARA1) and wk 8 (SARA2) after parturition. Rumen samples were taken on d 2 (SARA1/1, SARA1/2) and d 5 (SARA2/1, SARA2/2) during each SARA week. Wk 4 was considered as Pre-SARA1, wk 7 as Post-SARA1, and wk 10 and 12 as Post-SARA2.

³Statistical analyses were conducted on log₁₀-transformed data for Faith's Phylogenetic diversity, original data for Chao1 and Box-cox data for the rest. Presented means are original values prior to transformation.

⁴Int: Interaction effect of treatment and stage.

Table 4-2. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on the relative abundances (%) of the main phylum of rumen liquid microbiome in lactating dairy cows

Item	Treatment ¹				Stage ²					SEM	P- values ³					
	Control	SCFPa	SCFPb-1X	SCFPb-2X	Pre-SARA1	SARA1	Post-SARA1 (wk 7)	SARA2	Post-SARA2 (wk 10)		Treat	Stage	Treat × Stage	Contrasts ⁴		
														1	2	3
Actinobacteria	0.39	0.44	0.46	0.41	0.56 ^a	0.32 ^b	0.53 ^a	0.32 ^b	0.46 ^{ab}	0.04	0.74	<0.001	0.01	0.71	0.51	0.42
Bacteroidetes	40.22	38.53	37.45	41.62	40.27	40.15	38.96	39.63	38.27	1.37	0.18	0.86	0.46	0.19	0.27	0.38
Firmicutes	48.52	51.28	51.80	49.08	50.95 ^{ab}	47.48 ^{abB}	52.01 ^{ab}	47.13 ^b	53.51 ^{aA}	0.24	0.44	0.02	0.58	0.47	0.37	0.23
Proteobacteria	4.81	4.20	4.61	3.88	2.98 ^b	6.67 ^a	3.55 ^b	6.70 ^a	3.19 ^b	0.39	0.58	<0.001	0.95	0.26	0.53	0.57
Fibrobacteres	0.26	0.27	0.18 ^B	0.28 ^A	0.38 ^a	0.15 ^b	0.33 ^a	0.13 ^b	0.34 ^a	0.04	0.05	<0.001	0.65	0.21	0.22	0.07
Tenericutes	0.29	0.28	0.21	0.32	0.64 ^a	0.10 ^b	0.46 ^a	0.07 ^b	0.57 ^a	0.03	0.16	<0.001	0.25	0.14	0.21	0.16
F:B ⁵	1.22	1.36	1.37	1.20	1.28	1.20	1.35	1.20	1.41	0.28	0.35	0.36	0.56	0.33	0.13	0.87

^{a-b}Means in a row with different superscripts among Treatments are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts among Treatments tended to be different ($0.05 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on log-transformed data for Actinobacteria and Firmicutes, original data for Bacteroidetes, and Box-cox data for the rest. Presented means are original values prior to transformation.

⁴Contrasts: 1 = Control vs. SCFP, 2 = SCFPa vs. SCFPb, 3 = SCFPb-1X vs. SCFPb-2X.

⁵F:B: Ratio of Firmicutes: Bacteroidetes.

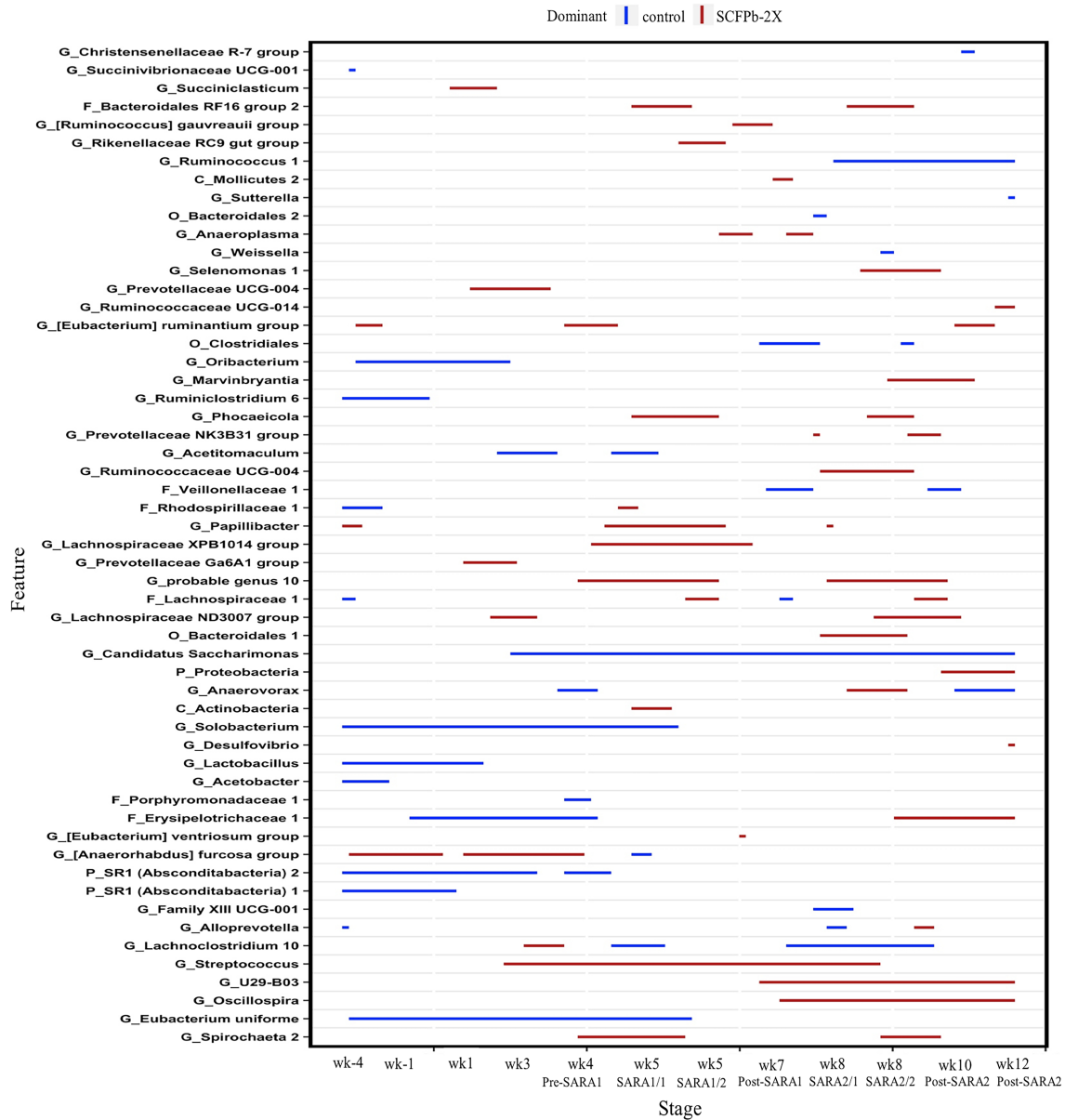


Figure 4-3. Differentially abundant taxa in rumen liquid microbiota in control vs. SCFPb-2X. The graph summarizes data that were generated using Metagenomic Longitudinal Differential Abundance (MetaLonDA) method. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. X axes represent stage relative to parturition. Y axes represent taxa that were promoted by control (blue lines) and SCFPb-2X (red lines) groups during corresponding stages.

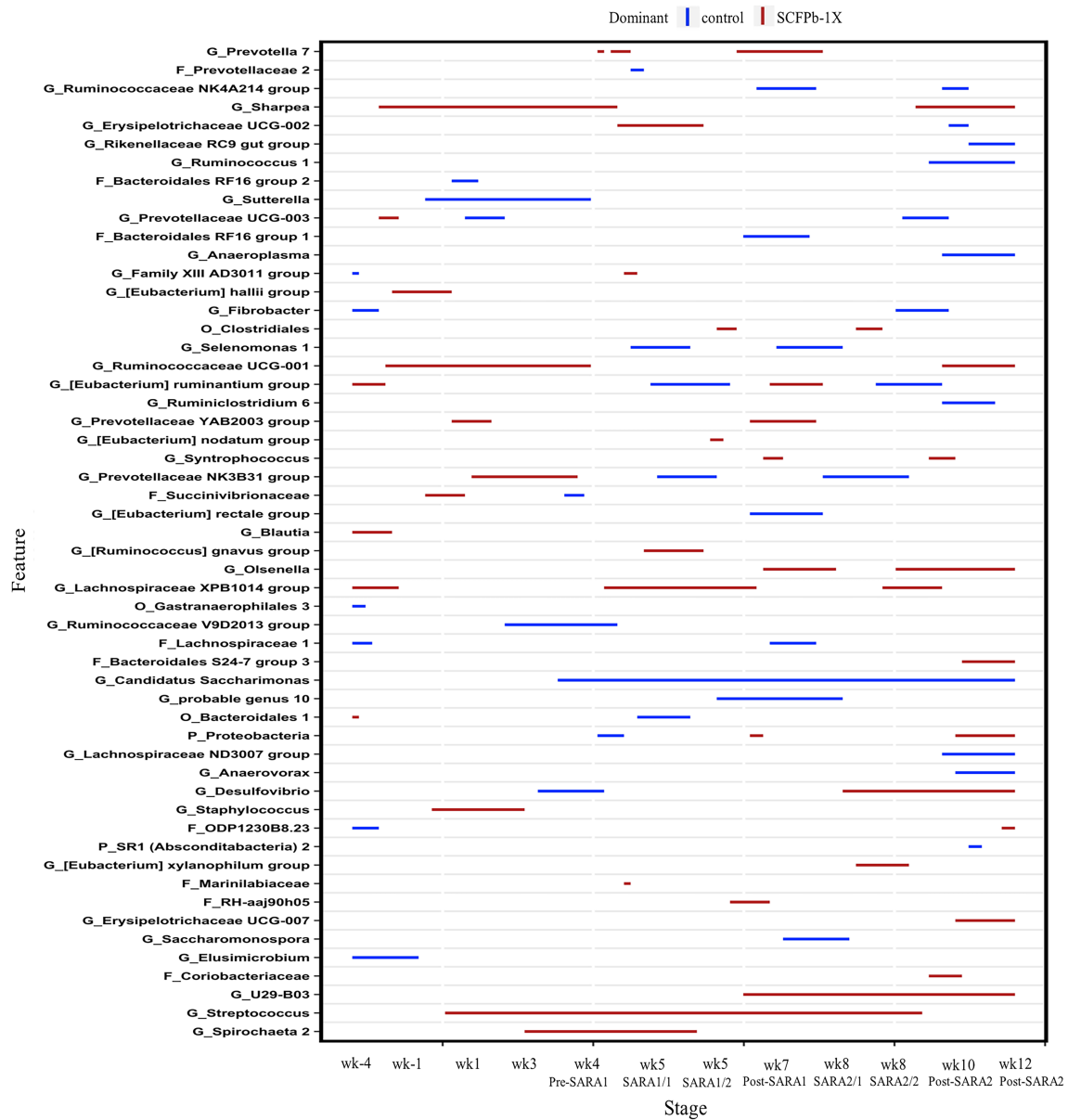


Figure 4-4. Differentially abundant taxa in rumen liquid microbiota in control vs. SCFPb-1X. The graph summarizes data that were generated using Metagenomic Longitudinal Differential Abundance (MetaLonDA) method. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. X axes represent stage relative to parturition. Y axes represent taxa that were promoted by control (blue lines) and SCFPb-1X (red lines) groups during corresponding stages.

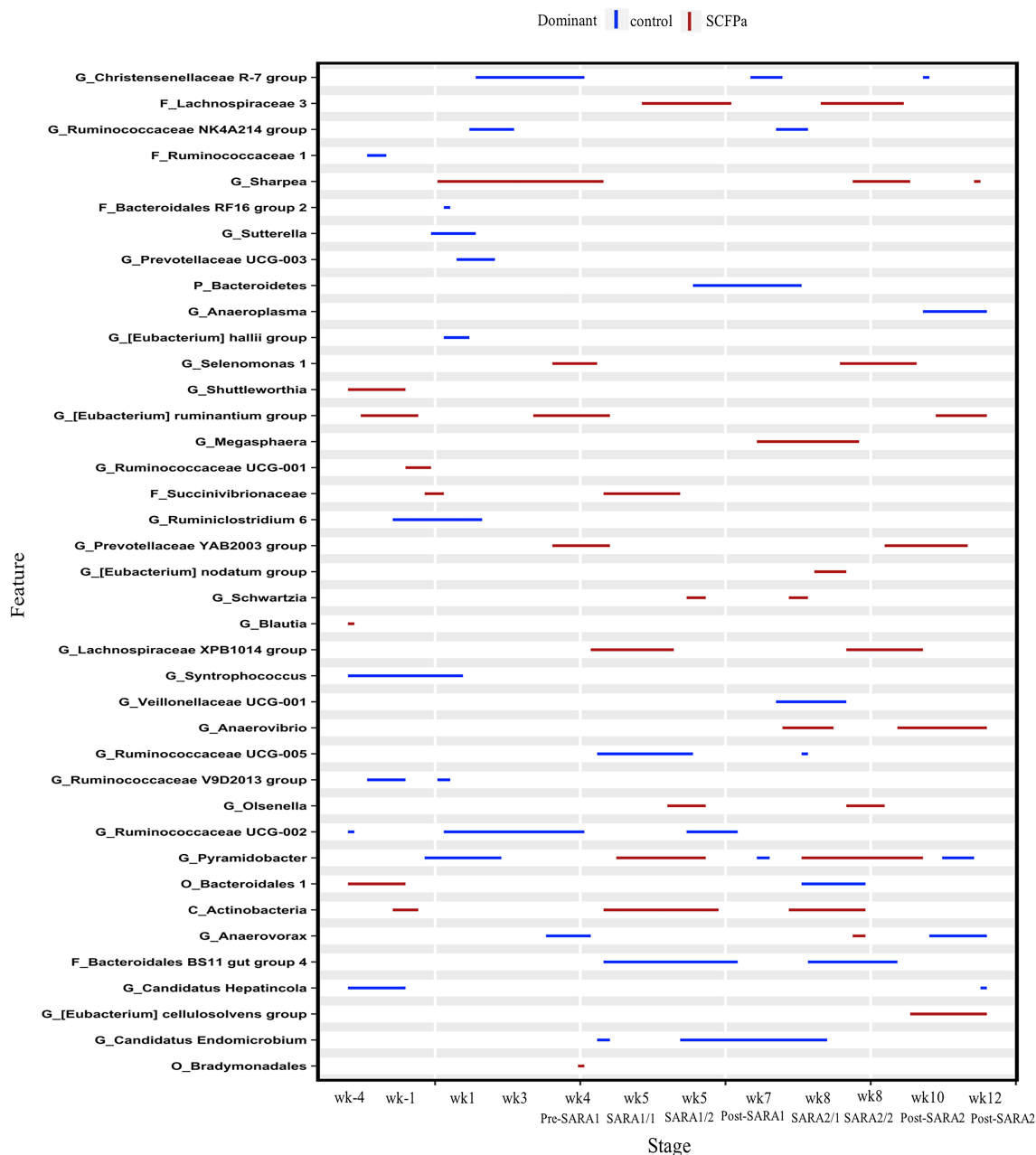


Figure 4-5. Differentially abundant taxa in rumen liquid microbiota in control vs. SCFPa.

The graph summarizes data that were generated using Metagenomic Longitudinal Differential Abundance (MetaLonDA) method. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. X axes represent stage relative to parturition. Y axes represent taxa that were promoted by control (blue lines) and SCFPa (red lines) groups during corresponding stages.

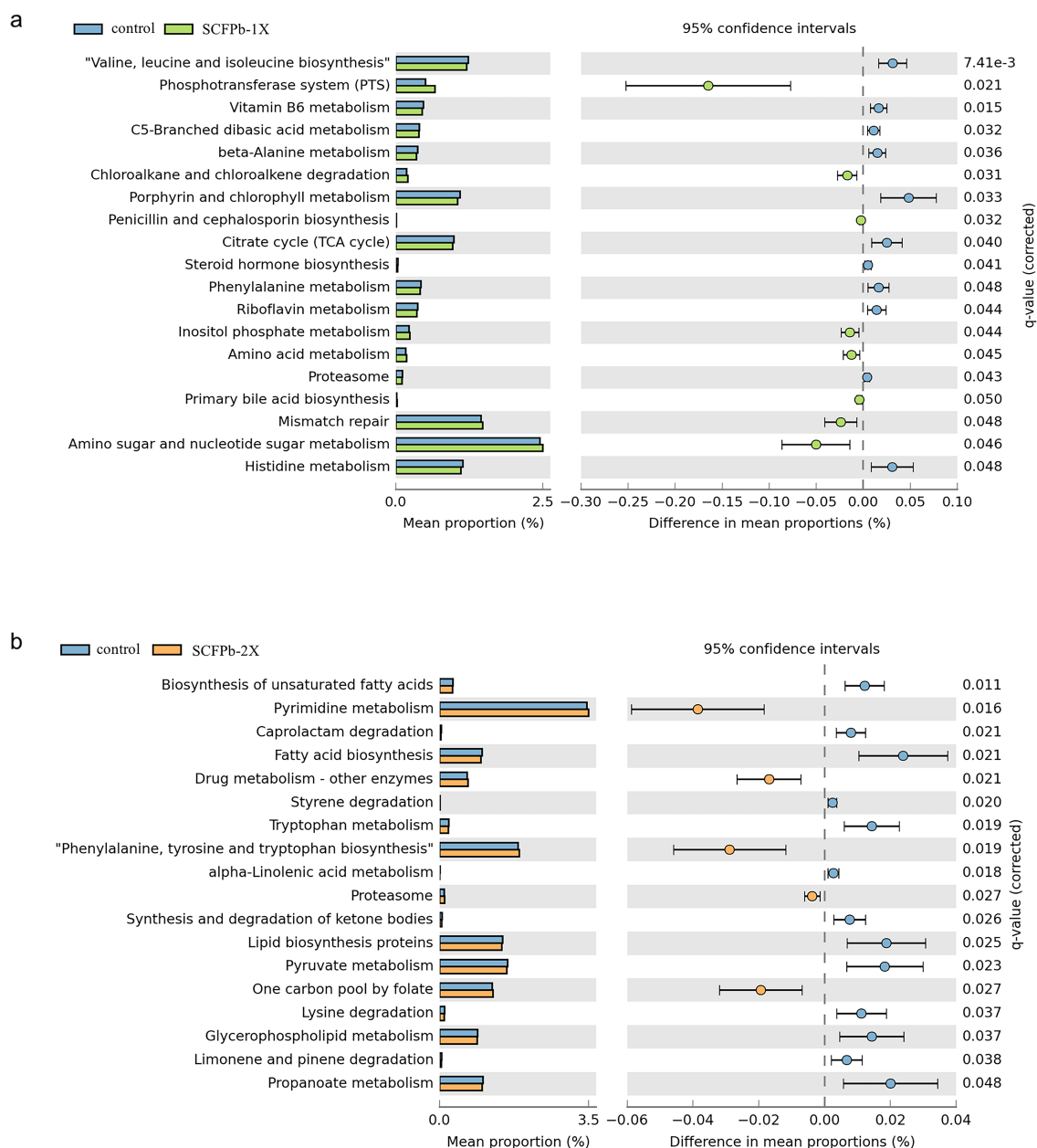


Figure 4-6. Differences in predicted functions of rumen liquid microbiota between control and SCFPb-1X and 2X groups. Functionalities of rumen liquid microbiota were predicted using CowPi. Output was analyzed using STAMP following log₂ transformation and False Discovery Rate correction. Significant differences were considered as $P < 0.05$. a. control vs. SCFPb-1X; b. control vs. SCFPb-2X.

The SCFPb-1X treatment increased the relative abundance of several taxa including, that of members of Ruminococcaceae, Prevotellaceae, Lachnospiraceae, *Sharpea*, *Olsenella*, *U29-B03*, *Streptococcus* and *Spirochaeta* compared to the Control treatment during SARA challenges ($P < 0.05$). A comparison of the Control and SCFPa treatments revealed that the relative abundances of 70 bacterial taxa were increased by one of the treatments (Figure 4-5). The SCFPa treatment increased the relative abundances of members of Lachnospiraceae, Succinivibrionaceae, Prevotellaceae, *Sharpea*, *Selenomonas*, *Megasphaera*, *Anaerovibrio*, *Pyramidobacter*, and *Olsenella* compared to the Control treatment during the SARA challenges ($P < 0.05$).

4.4.3 Predicted functionality of rumen liquid microbiota

The predicted functionality of microbiota in rumen liquid digesta revealed a total of 150 of 254 endogenous third-level KEGG rumen microbial metabolic pathways. Comparisons of differences in the predicted functionality of microbiota in rumen liquid digesta between Control and SCFP treatments during both non-SARA and SARA stages showed that the SCFPb-2X treatment reduced 2 predicted carbohydrate metabolic pathways, including “propanoate metabolism” and “pyruvate metabolism”, increased 1 predicted amino acid pathway “phenylalanine, tyrosine and tryptophan biosynthesis”, and inhibited 6 predicted lipid including “biosynthesis of unsaturated fatty acids”, “fatty acid biosynthesis”, “glycerophospholipid metabolism”, “lipid biosynthesis proteins”, “synthesis and degradation of ketone bodies”, “alpha-linolenic acid metabolism” metabolic pathways compared to the Control treatment ($P < 0.05$, Figure 4-6b). The SCFPb-1X treatment did not impact the predicted rumen lipid digesta metabolic

pathways, but it increased 2 predicted carbohydrate pathways including “amino sugar and nucleotide sugar metabolism”, “inositol phosphate metabolism” metabolic pathways and 1 predicted amino acid “amino acid metabolism” metabolic pathway, and reduced 2 predicted carbohydrate pathways including “C5-branched dibasic acid metabolism” and “citrate cycle (TCA cycle)” and 3 predicted amino acids metabolic pathways including “valine, leucine and isoleucine biosynthesis”, “histidine metabolism” and “phenylalanine metabolism” compared to the Control treatment ($P < 0.05$, Figure 4-6a). No differences were observed between the Control and SCFPa treatment in the predicted functionality of microbiota in rumen liquid digesta.

4.4.4 Association of SCFP supplementation and SARA challenges with Co-occurrence patterns of rumen liquid microbiota

Correlation network analysis was performed to evaluate potential associations of SCFP supplementation and SARA challenges with co-occurrence patterns of rumen liquid microbiota (Figures 4-7, 4-8). The SARA challenges reduced the total number of significant associations among different microbial taxa. Although the proportions of abundant bacterial phyla were not different within each SCFP treatment group during SARA stages (Table 4-2), the relative degrees of connectedness (total number of positive and negative edges observed for each phylum divided by its relative abundance in the community) varied greatly among SCFP treatments (Figures 4-7a and 4-7b).

Averaged across non-SARA stages, the degrees of positive and negative connections were higher in SCFP supplemented cows, compared to Control cows (Figure 4-7a). Of all major phyla, Bacteroidetes showed the highest degree of positive connections in SCFP

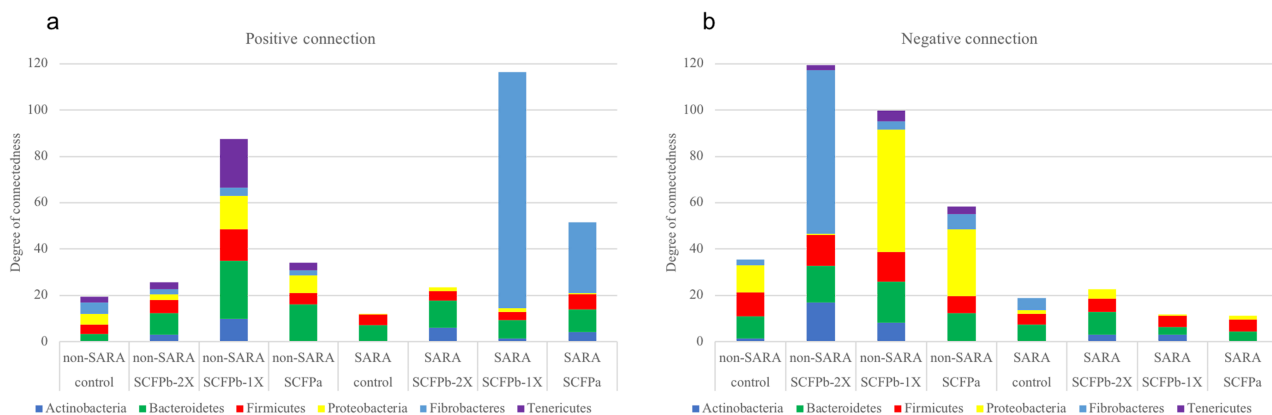


Figure 4-7. Microbial interaction networks. The degree of connections for each phylum was normalized by dividing the total number of positive and negative edges observed for each phylum by their relative abundance in the community. a) Normalized positive connections for the dominant bacteria phyla within each treatment group during non-SARA (Pre-SARA1, Post-SARA1 and Post-SARA2) and SARA (SARA1/1, SARA1/2, SARA2/1, SARA2/2) stages. b) Normalized negative connections for the dominant bacteria phyla with each treatment group during non-SARA and SARA stages.

supplemented cows. In descending order, Bacteroidetes, Firmicutes had a high number of positive connections in SCFPb-2X supplemented cows, whereas Tenericutes, Proteobacteria and Firmicutes had a high number of positive connections in SCFPb-1X supplemented cows, and Proteobacteria and Firmicutes had high number of positive connections in cows on the SCFPa treatment (Figure 4-7a). In contrast, in Control cows, the number of positive connections was equal for the Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes phyla. Fibrobacteres contributed to the largest number of negative connections in the SCFPb-2X treatment, whereas in the SCFP-1X and SCFPa treatments, Proteobacteria was the major contributor to negative connections (Figure 4-7b). Negative connections in Control cows were contributed equally by the phylum Proteobacteria, Firmicutes and Bacteroidetes.

Averaged across SARA challenges, Bacteroidetes had the largest number of positive connections for cows in the Control and SCFPb-2X treatments, whereas Fibrobacteres had the highest number of positive connections in the SCFPa and SCFPb-1X treatments. The number of positive connections was 2.5 times and 6 times greater in SCFPb-1X and SCFPa treatments, respectively, compared to the Control treatment. All treatment groups showed a smaller number of negative connections among bacterial taxa during the SARA stages compared to the non-SARA stages.

Hub taxa were identified as taxa with the highest number of positive or negative connections with other members of the community (> 15 connection; Figure 4-8). The SARA challenges reduced the number of hub taxa in all treatments. During non-SARA stages, Control cows had several negatively connected hub taxa from Bacteroidetes (4 OTUs belonging to genus *Prevotella* and 1 OTU from Rikenellaceae RC9 family) and from Firmicutes (2 OTUs of Christensenellaceae R-7 family, 2 OTUs of Ruminococcaceae family and 1 OTU of genus *Ruminiclostridium*) (Figure 4-8a). The SCFPb-2X treatment had negatively connected hub taxa from the phyla Bacteroidetes (7 OTUs belonging to *Prevotella* genus or Prevotellaceae family), Firmicutes (2 OTUs of Ruminococcaceae family, 2 OTUs of Christensenellaceae R-7 family, 1 OTU of Lachnospiraceae NK3A20 family, and 1 OTU of each of *Weissella* and *Ruminiclostridium* genera) and Fibrobacteres (1 OTU from *Fibrobacter* genus) (Figure 4-8c). In contrast, the SCFPb-1X treatment resulted in a combination of negatively and positively connected hub taxa from the phyla Proteobacteria (1 negatively connected OTU of Succinivibrionaceae UCG-001 family), Bacteroidetes (9 OTUs including 5 negatively connections from the *Prevotella* genus, 4 positively connections of

Bacteroidales RF16 class, and 2 positive connections from Rikenellaceae RC9 family), and Firmicutes (1 positively connected OTU of Ruminococcaceae NK4A214 family, 3 positively connected OTUs of Christensenellaceae R-7 family, 2 positively connected OTUs of Lachnospiraceae NK3A20 family, 2 negative connected OTUs of *Mitsuokella* genus, and 1 negative connected OTU from genus *Sharpea*) (Figure 4-8e). Similar to the SCFPb-1X treatment, the SCFPa treatment resulted in a combination of negatively and positively connected hub taxa. However, the number of these hub taxa was less than that of the SCFPb-1X treatment (18 vs. 40). The hub taxa in the SCFPa treatment were from the phyla Proteobacteria (1 negatively connected OTU of Succinivibrionaceae UCG-001 family), Bacteroidetes (5 negatively and 10 positively connected ASVs from genus *Prevotella*), and Firmicutes (1 negative connected OTU belonging to *Ruminococcus* genus) (Figure 4-8j).

During the SARA challenges, hub taxa in all treatments were members of the Bacteroidetes and Firmicutes phyla. The Control treatment was characterized by 3 negatively connected and 2 positively connected taxa belonging to genus *Prevotella* and 1 negatively connected taxon of *Ruminococcus gauvreauii* (Figure 4-8b). The SCFPb-2X treatment was characterized by 7 negatively taxa and 1 positively connected taxon from genus *Prevotella*, and 2 negatively connected taxa, including one from *Ruminococcus gauvreauii* and one from the Erysipelotrichaceae UCG-002 family (Figure 4-8d). The SCFPb-1X treatment resulted in two negatively connected taxa from the *Ruminococcus* genus, one negatively connected taxon of the *Dialister* genus, and one positively connected taxon from genus *Prevotella* (Figure 4-8f). Finally, the SCFPa treatment resulted in one positively and two negatively connected taxa from the Lachnospiraceae

family, 2 negatively connected taxa from the Ruminococcaceae family, and three positively connected taxa belonging to genus *Prevotella* (Figure 4-8h).

4.4.5 Correlation between hub Taxa, biodiversity metrics of rumen liquid microbiota and rumen fermentation characteristics

In order to evaluate the influence of hub OTUs, the relationships among hub OTUs and biodiversity metrics of rumen liquid microbiota and rumen VFAs and ammonia concentrations that were presented in our companion paper (Khalouei et al., 2020) were determined. As shown in Figures 4-9a and 4-9b, a group of ASVs from Bacteroidetes, including the Bacteroidales RF16 family and the Rikenellaceae RC9 family, ASVs from Firmicutes, including the Christensenellaceae R-7 family, OTUs from the Ruminococcaceae NK4A214 family and unclassified Ruminococcaceae family, as well as one OTU from genera *Fibrobacter* and *Prevotella 1* were negatively correlated with the concentrations of propionate, butyric and lactate in rumen liquid digesta. However, these taxa were positively correlated with the acetate and ammonia concentrations in rumen fluid and the α -, β -diversity metrics of the microbiota in rumen liquid. Another group of OTUs from Bacteroidetes, including members of the *Prevotella 7* and *Ruminococcus gauvreauii* genera, from Firmicutes, including members of *Sharpea*, *Dialister* and *Mitsuokella* genera, and from Proteobacteria, including the Succinivibrionaceae UCG-001 family were positively correlated with propionate concentrations in rumen fluid, but negatively correlated with acetate and ammonia concentrations in rumen fluid and the α -, β -diversity metrics of microbiota in rumen liquid digesta.

stages. a, b) control; c, d) SCFPb-2X; e, f) SCFPb-1X; j, h) SCFPa. Yellow color represents negative connections while blue represents positive connections with other community members.

4.5 Discussion

The diversity, richness and taxonomic composition of microbial communities affect their functional properties, such as robustness and resilience (Konopka, 2009). Also, interactions among microbiota are important to the diversity and stability of microbial communities. Due to high prevalence of SARA in dairy industry, more studies on the etiology of SARA are needed to minimize its adverse effects on animal gastrointestinal health. In this study, we determined the effects of grain-based SARA challenges and SCFP supplementation on diversity, composition, co-occurrence patterns and predicted functional potential of the microbiota in rumen liquid digesta.

4.5.1 Effects of grain-Based SARA challenges on diversity, composition and predicted functionality of rumen liquid microbiota

Our companion study (Khalouei et al., 2020) showed that during this trial, the SARA challenges resulted in a rumen pH depression between 5.2 and 5.6 was maintained for more than 180 min/d, which is a commonly accepted threshold of SARA (Gozho et al., 2007; Plaizier et al., 2012a). The SARA challenges included an increase in the dietary starch content from 17.6 to 27.9% DM and a reduction of the dietary NDF content from 55.4 to 48.1 % DM. (Khalouei et al., 2020; Guo et al., 2022). As a result, we expected that the relative abundances of fibrolytic and pH sensitive bacteria would decrease, while those of amylolytic and pH tolerant bacteria were expected to increase (Mao et al., 2013; Petri et al., 2013; Tun et al., 2020). Gram-negative bacteria shed the endotoxin LPS

during rumen pH depressions and can cause inflammation (Gozho et al., 2007; Plaizier et al., 2012a). Another companion manuscript (Guo et al., 2022) also reported that SARA increased the concentration of LPS in rumen liquid digesta from 5,012 to 63,596 EU/mL, and increased the blood plasma concentration of serum amyloid A (SAA) from 19.76 to 71.08 µg/mL. Therefore, we concluded that SARA was induced successively and that it caused inflammatory responses.

The reduction in richness and diversity of the microbiome of rumen liquid digesta during the SARA challenges agree with previous studies (Mao et al., 2013; Khafipour et al., 2016; Plaizier et al., 2017), and suggests that the robustness and stability of rumen bacterial community were reduced by these challenges. Rumen microbial members are essential for ruminants' feed utilization, including digestion and fermentation of fiber, and conversion of feed into to absorbable compounds (Firkins and Yu, 2015). Analysis of PERMANOVA and PERMDISP of Bray-Curtis distances of rumen liquid microbial communities also demonstrated that SARA challenges affected the taxonomic composition and beta diversity of the rumen liquid microbiota. As in previous studies, we found that Firmicutes, Bacteroidetes and Proteobacteria were the dominant phyla of rumen liquid microbiota (Kim et al., 2011; Tun et al., 2020). In contrast from earlier studies (Fernando et al., 2010; Mao et al., 2013; McCann et al., 2016; Tun et al., 2020), we observed the SARA challenges tended to reduce the relative abundance of Firmicutes while not affecting that of Bacteroidetes. Besides, no effect of SCFP treatment or SARA was observed on Firmicutes:Bacteroidetes ratio, indicating the feed efficiency may not be affected (Fernando et al., 2010; Jami et al., 2014). Meanwhile, we found that the

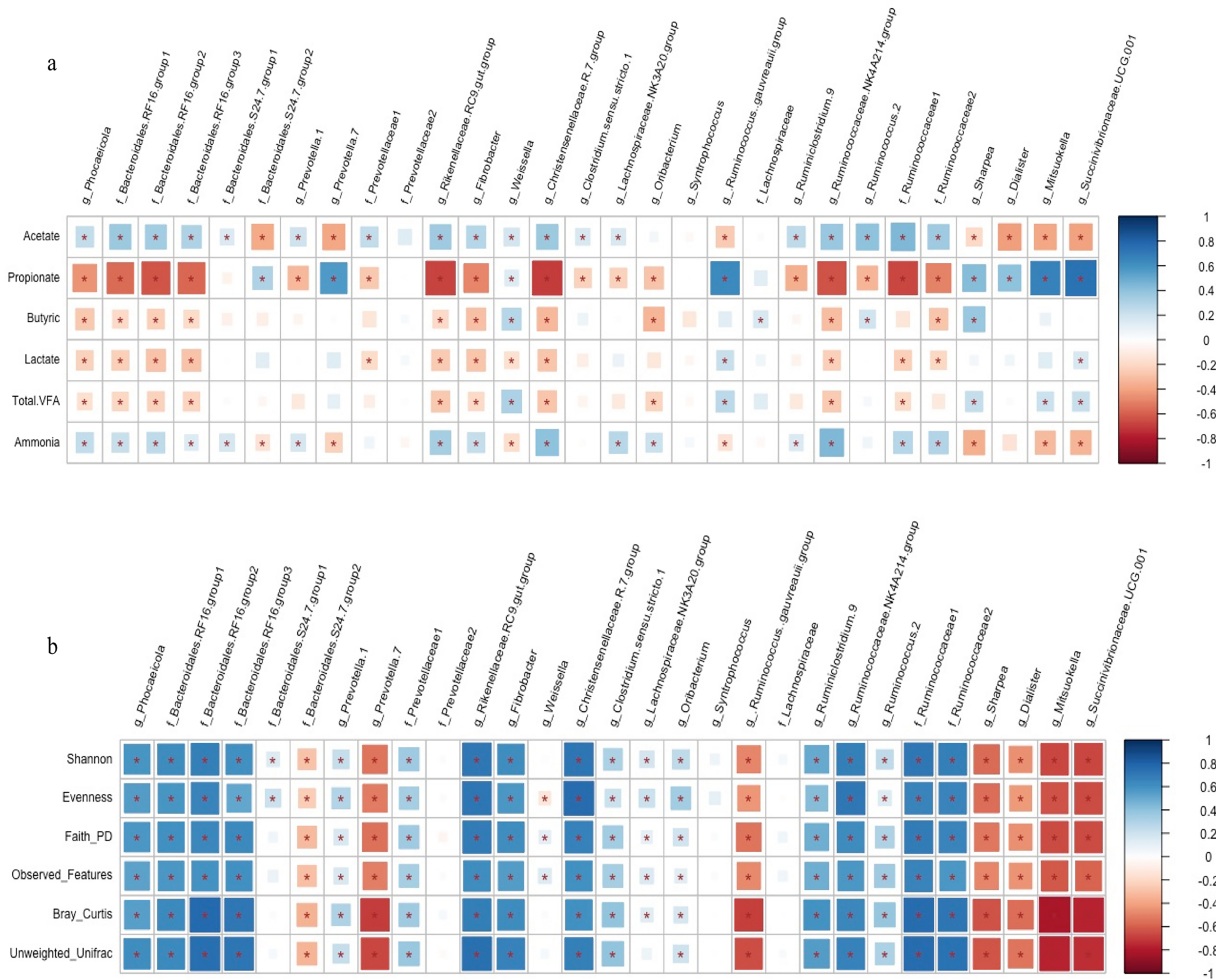


Figure 4-9. Correlation among hub taxa and rumen fermentation characteristics and biodiversity metrics of rumen liquid microbiota. Spearman's correlation coefficient was used to explore the relationships between the relative abundances of rumen liquid hub taxa and community α -diversity (Shannon, Evenness, Faith_PD and Observed_Features), β -diversity (Bray-Curtis dissimilarities and unweighted UniFrac distances) and rumen fermentation characteristics (VFA and ammonia concentrations). * Indicates $P < 0.05$, ** Indicates $P < 0.01$. The color ramp and the size of the squares indicate the type and strength of the Spearman's correlation coefficient (ρ): $\rho = 1$ showing strong positive correlation and $\rho = -1$ showing strong negative correlation between the two parameters.

abundance of Proteobacteria phyla increased during the SARA challenges, which contrasts with the findings of Tun et al., 2020 (Tun et al., 2020). Khafipour et al. (2009c) found that the relative abundance of Firmicutes in the rumen liquid digesta was increased in cows that responded severely to a grain-based SARA challenge, but not in cows that responded moderately to this challenge. This discrepancy among studies, may be due to the difference in dietary starch percentage, experimental design, sampling time and methods, sequence primers and the methodologies used in 16s rRNA sequencing (McCann et al., 2016; Tun et al., 2020). Several taxa of the Bacteroidetes are considered to be primary degraders of complex polysaccharides, as they are more efficient in fermenting these compounds compared with Firmicutes (El Kaoutari et al., 2013). It has also been reported that Bacteroidetes are more abundant in grain fed cows and during SARA challenges (Fernando et al., 2010; McCann et al., 2016; Tun et al., 2020). Prevotellaceae is one of the most abundant family in Bacteroidetes, some members of which can utilize several substrates such as starch, protein and peptides, and generate a wide range of end products, including acetate, succinate, and propionate (Russell and Rychlik, 2001; Li and Guan, 2017). This may be explained by the most dominant genus *Prevotella* of this family that contains a wide range of functional capacities including cellulolytic, amylolytic and fibrolytic activities (Bekele et al., 2010; Tun et al., 2020; Xue et al., 2020). The relative abundance of this genus is positively related to concentration of total VFAs while negatively related to rumen pH (Zhang et al., 2022). In our study, several ASV that were recognized as *Prevotella* responded differently to SARA. One of the most abundant of these taxa, *Prevotella* 7, was positively correlated to the concentration of propionate in rumen digesta, and negatively related to the richness and

evenness of the rumen liquid microbial community. Its relative abundance increased during both SARA challenges, indicating its correlation with SARA. Besides, hub taxa in both non-SARA and SARA stages of our study mostly belonged to *Prevotella*, which has both positive and negative connections with other members of the rumen liquid microbial community.

Rumen pH depressions during SARA challenges affect the growth of microorganism's species that are sensitive to acidic environment. The growth of several fibrolytic bacteria, such as members from *Christensenellaceae R-7 group* and *Lachnospiraceae* are strictly anaerobic, and their cell growth and enzyme activities are suppressed when the proportion of fiber in diet is decreased (Zhou et al., 2015). This was also observed in our study. The Family *Ruminococcaceae* contains major fibrolytic bacteria. The relative abundances of members from this family including unclassified *Ruminococcaceae* and *Ruminococcus* are decreased by SARA (Zhang et al., 2022). In partial agreement, we observed that grain-based SARA reduced the abundances of the abundant fibrolytic genera *Ruminococcaceae NK4A214 group*, *Papillibacter*, *Christensenellaceae R-7 group* and unclassified *Lachnospiraceae*. Several members of *Succinivibrionaceae* can produce succinate, which is the precursor of the propionate that is a substrate for gluconeogenesis (Li and Guan, 2017). We observed that the relative abundance of *Succinivibrionaceae UCG-001* was positively correlated with the rumen concentrations of propionate, lactate and total VFA, and negatively correlated with the richness and evenness of the rumen microbial community. In our study, the SARA challenges increased the rumen concentration of propionate (Khalouei et al., 2020), as

well as the relative abundance of *Succinivibrionaceae* UCG-001. This shows the adverse effect of SARA on richness and diversity of rumen microbiota.

Several amylolytic microorganisms, including the lactate producer *Streptococcus bovis*, are tolerant to the rumen pH depressions caused by SARA (McCann et al., 2016; Clemmons et al., 2019). The reason that we did not find an effect of SARA on the relative abundance of this bacterium may be due to its low relative abundance and SARA conducted in our study was not as severe as in other studies.

Several members of the family Lachnospiraceae are producers of butyrate (Meehan and Beiko, 2014). The production of butyrate provides energy for some microbes and host epithelial cells (Freetly et al., 2020). It has been reported that the concentrations of butyrate in the rumen are positively related to feed efficiency, which may be due to the increase in the supply of energy to the host animal (Guan et al., 2008). However, other studies reported that a higher abundance of this family may increase butyrate metabolism and reduce feed efficiency (Myer et al., 2015; Li and Guan, 2017). In the current study, we detected that the relative abundance of unclassified Lachnospiraceae was positively correlated with the butyrate concentration in the rumen. This may demonstrate the large contribution of this family to ruminal butyrate production. Due to large number of genera of this family that produce butyrate, and the limitation of 16s rRNA sequencing in identifying taxa at the species level, the relationships between this family and feed efficiency needs further study.

Previous studies have shown that inefficient dairy cows have higher nitrogen metabolism activities compared to efficient cows (Li and Guan, 2017). Similarly, from the functional prediction of rumen liquid microbiota, we found that predicted “nitrogen

metabolism” was inhibited by SARA, including “phenylalanine, tyrosine and tryptophan biosynthesis”, “alanine, aspartate and glutamate metabolism”, “lysine biosynthesis”, “phenylalanine metabolism”, “glycine, serine and threonine metabolism” and “arginine and proline metabolism”. Besides, rumen ammonia-nitrogen was reduced by the SARA challenges (Khalouei et al., 2020). It may indicate that the proteolytic bacteria were suppressed during SARA as starch content increases, and the ammonia from protein degradation by these proteolytic bacteria, which is the main source of nitrogen for fibrolytic bacteria were also decreased (Leng and Nolan, 1984; Morrison and Mackie, 1996; Genzebu and Tesfay, 2015). This suggests that the availability of substrates for microbial protein synthesis is decreased.

4.5.2 Effects of SCFP supplementation on diversity, composition and predicted functionality of rumen liquid microbiota

Several SCFP have been used to improve the rumen function and feed utilization, as they are rich in several constituents such as vitamins, short-chain fatty acids (SCFAs), microbial cell fractions, functional proteins, extracellular polysaccharides, cell lysates, antioxidants and β -glucans that promote the growth of lactate-utilizing bacteria, cellulolytic bacteria and fungi in the rumen, and stabilize the ruminal pH (Callaway and Martin, 1997; Poppy et al., 2012; Zhu et al., 2017; Wegh et al., 2019; Tun et al., 2020). Studies have reported that supplementation with SCFP helps to maintain rumen pH during high-starch diet feeding and under grain-based SARA challenges (Allen and Ying, 2012; Li et al., 2016; Dias et al., 2018). Our companion papers showed that SCFP supplementation, and especially SCFPb-2X, attenuates the decrease of rumen pH, and NDF digestibility, and decreased the rumen concentration of free LPS and the acute

phase response, caused by SARA challenges (Khalouei et al., 2020; Guo et al., 2022).

This indicates that SCFP attenuates several adverse effects of SARA.

Previous studies suggested that SCFP improves milk production and feed efficiency and stabilizes rumen fermentation by enhancing the growth of fiber-digesting and lactic acid-utilizing bacteria (Poppy et al., 2012; Zhang et al., 2013; Brainard et al., 2016; Zhu et al., 2017). In addition, Tun et al. (2020) demonstrated that SCFP (XPC) attenuate the reduction of richness and diversity and changes of the β -diversity of rumen microbiota during SARA challenges (Tun et al., 2020). Rumen microbial members have their specific substrates and vary in functionality, a more diverse community may imply more efficient use of feed, as the functionality of taxa that are reduced by nutritional challenges may be replaced by taxa with similar functionality that are less affected by these challenges (Mao et al., 2013; Henderson et al., 2015). In agreement, the current study shows that cows on the SCFPb-2X treatment tended to have higher evenness of the rumen microbiota than cows on the control treatment during the first SARA challenge (SARA1). This shows that the dose effect of SCFPb, in that only in 2X supplementation can be sufficient to reduce the microbial variation caused by SARA challenges.

Additionally, our results show that the changes of β -diversity of rumen liquid microbiota were less in SCFP treatment groups than in the Control treatment group, which indicates the effect of the SCFP supplementation on the robustness and stability of the microbial community. Our study also found that during SARA challenges, SCFPb-2X stimulated the growth of several fibrolytic bacteria, including *Ruminococcaceae NK4A214 group*, *Papillibacter*, *Christensenellaceae R-7 group* and unclassified Lachnospiraceae, and the lactate utilizer *Selenomonas* (Loor et al., 2016) during SARA challenges compared with

the control treatment. In contrast, the relative abundances of *Ruminococcaceae NK4A214 group*, *Christensenellaceae R-7 group* and unclassified Lachnospiraceae were negatively correlated with ruminal concentrations of propionate and butyrate that were increased during SARA, while being positively correlated with the richness and evenness of the microbial community. Although SCFPb-2X supplementation contributed to maintaining the diversity of the rumen microbiota during SARA challenges, this did not affect the rumen concentrations of propionate and butyrate. In addition, the SCFPa treatment increased the relative abundances of *Selenomonas* and *Megasphaera*, which are main lactate utilizers (Loor et al., 2016). The latter demonstrates its effect on preventing the accumulation of lactic acid and severe rumen acidosis.

In our study, supplementation with SCFP attenuated the fluctuations in the relative abundances of abundant Bacteroidetes and Firmicutes phyla that resulted from the SARA challenges. However, only SCFPb-2X attenuated this fluctuation of Proteobacteria and Tenericutes. Although the relative abundances of the Proteobacteria and Tenericutes phyla were lower than those of the Bacteroidetes and Firmicutes phyla, Proteobacteria and Tenericutes showed strong connections with other taxa, which made them rumen keystone members of the microbial community in rumen liquid. Thus, SCFPb-2X stabilizes populations of both foundation and keystone members during grain-based SARA conductions. In addition to the higher evenness during SARA1, SCFPb-2X also improved the robustness and stability of the structure of the rumen liquid microbial community during SARA challenges in our study. A more diverse community with complex biotic interactions among commensal microbes has a stronger stability and capability to withstand exogenous perturbations, and the community has higher and more

diverse abilities to utilize feed compounds and generate more diverse metabolic products compared to a less diverse community (Lozupone et al., 2012; Konopka et al., 2015; Li and Guan, 2017; Derakhshani et al., 2020). We observed that SCFPb-1X and SCFPa supplementation promoted the positive connectedness among members of microbiota in liquid rumen digesta during both SARA and non-SARA experimental stages. Both SCFPb-2X and SCFPa supplementation increased the diversity of hub ASVs during both non-SARA and SARA stages, while SCFPb-1X only increased the diversity of hub ASVs during non-SARA stages. This indicates that SCFPb-1X has a smaller effect on the composition of the rumen liquid microbiota. Supplementation with SCFPb-2X promoted populations of taxa (*Prevotella* and *unclassified Bacteroidales RF16*) that showed strong associations with biodiversity metrics mostly belonged to Bacteroidetes, whereas SCFPb-1X and SCFPa promoted taxa that mostly belonged to Firmicutes during SARA challenges. In contrast, SCFPb-2X is more beneficial in increasing rumen fermentation when high-grain diets are supplied.

An increase in the intake of starch increases the production and absorption of propionate (Sutton et al., 2003). It has been reported that SCFP (XP yeast culture) reduces the ruminal digestibility and fermentation of starch when cows receive large amounts of starch (Allen and Ying, 2012). In agreement, we also observed that the SCFPb-2X treatment had a lower predicted “propanoate metabolism” compared with the control treatment during SARA, which demonstrates the effect of SCFPb with higher dose on reducing starch digestibility. Furthermore, SCFPb-1X reduced several nitrogen metabolism pathways, including “valine, leucine and isoleucine degradation”, “beta-alanine metabolism”, “phenylalanine metabolism” and “histidine metabolism”.

Supplementation with SCFPb-2X, however, reduced “tryptophan metabolism” and “lysine degradation”. Valine, leucine and isoleucine are important in microbial protein synthesis, and microbial protein provides most precursors for the synthesis of milk protein in the mammary gland (Allison et al., 1966; Xue et al., 2020). Xue et al. (2020) reported that “valine, leucine and isoleucine degradation”, “lysine degradation” and “phenylalanine metabolism” were enriched in the microbiome of cows with low milk protein yields. (Xue et al., 2020). Therefore, SCFPb supplementation may help to decrease the degradation of the microbial protein and promotes more microbial protein synthesis in the rumen during grain-based SARA challenges.

4.6 Conclusions

Two subsequent grain-based SARA reduced the richness and diversity and changed the β -diversity of the microbial community in rumen liquid. Supplementation with SCFPb attenuated these effects compared to the Control treatment. The relative abundances of the predominate phyla Firmicutes, Bacteroidetes, Proteobacteria and Tenericutes in rumen liquid microbiota experienced fluctuations during SARA challenges and the recovery of these challenges. Supplementation with SCFP, and especially SCFPb-2X, reduced these fluctuations. Grain-based SARA challenges also reduced the biotic interaction between microbes, hub taxa diversity and the robustness of the microbiota in rumen liquid digesta. Supplementation with SCFP also increased the positive connectedness among rumen liquid microbial members and the diversity of hub taxa during both SARA and non-SARA stages. Grain-based SARA challenges reduced the growth of several fibrolytic bacteria, whereas supplementation with SCFP, and

especially that with SCFPb-2X increased the relative abundances of several fibrolytic and lactate utilizing bacteria, stimulated fiber degradation and reduced lactate accumulation. Grain-based SARA challenges increased the rumen concentration of the propionate precursor succinate. Grain-based SARA challenges also reduced several amino acids synthesis pathways, which may reduce the microbial protein synthesis and milk protein synthesis. Supplementation with SCFPb reduced the predicted degradation of some amino acids and it might help to improve microbial protein synthesis. And also, supplementation with SCFPb-2X reduced predicted “propionate metabolism” so that may decrease the starch digestibility. Our results indicate that grain-based SARA challenges altered the microbial environment in rumen liquid digesta and SCFPb-2X has a larger effect on attenuating negative effects of SARA challenges than SCFPa and SCFPb-1X. Due to the limitation of the 16s rRNA sequencing to classify taxa at the species level and of the databases for rumen microbiome when using CowPi to predict the microbial functionality, further metagenomic assembly based (MAGs) approaches are needed to investigate the association among microbes, and the gastrointestinal health of the host animal, especially when the animals are exposed to nutritional and physiological challenges.

CHAPTER FIVE

MANUSCRIPT THREE

Postbiotics from *Saccharomyces cerevisiae* fermentation stabilize rumen solid microbiota during grain-based subacute ruminal acidosis (SARA) challenges in lactating dairy cows.

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5.1 Abstract

High yielding dairy cows commonly receive high-grain diets to meet their high energy requirements. However, this can cause subacute ruminal acidosis (SARA), which is accompanied by systemic inflammation and rumen dysbiosis. Postbiotics that provide metabolites for a wide range of bacteria have been shown to attenuate these adverse effects of SARA. This study determined the effects of two commercially available postbiotics from *Saccharomyces cerevisiae* fermentation on rumen solid microbiota in lactating dairy cows subjected to a repeated grain-based SARA challenges. A total of 32 rumen-cannulated lactating dairy cows were randomly assigned into four treatments: without SCFP (Control), 14 g/d Original XPC (Diamond V, Cedar Rapids, IA.) (SCFPa), 19 g/d NutriTek (Diamond V, Cedar Rapids IA) (SCFPb-1X), and 38 g/d NutriTek (SCFPb-2X,) from 4 wk before until 12 wk after parturition. Grain-based SARA challenges were conducted during wk 5 (SARA1) and wk 8 (SARA2) after parturition by

replacing 20% DM of the base total mixed ration (TMR) with pellets containing 50% ground barley and 50% ground wheat. Rumen solid samples were collected weekly, with the exception that during SARA challenges these samples were collected twice weekly. The DNA of rumen solid digesta was extracted and subjected to V3-V4 16S rRNA gene sequencing. The composition, predicted function and co-occurrence patterns of the rumen solid microbiota were compared between non-SARA (Pre-SARA1, wk 4; Post-SARA1, wk 7; and Post-SARA2, wk 10 and 12) and SARA stages (SARA1/1, SARA1/2, SARA2/1, SARA2/2), as well as among treatment groups.

Both SARA challenges reduced the richness and diversity of the microbiota in rumen solid digesta, and the relative abundances of the phylum Fibrobacteres. Supplementation with SCFP promoted the growth of several fibrolytic bacteria including *Lachnospiraceae* UCG-009, *Treponema*, *unclassified Lachnospiraceae*, *unclassified Ruminococcaceae* during the SARA challenges. These challenges also reduced the degree of positive connectiveness and the numbers of hub taxa in solid rumen digesta. The SCFPb treatment increased positive interactions among microbial members of those digesta, as well as the number of hub taxa during both SARA and non-SARA stages. The SCFPb-2X treatment prevented changes in the network characteristics including the number of components, clustering coefficient, modularity, positive edge percentage and edge density of microbiota in solid rumen digesta during the SARA challenges. These challenges reduced predicted carbohydrates metabolism and nitrogen metabolism in microbiota, whereas SCFP supplementation attenuated this reduction. Therefore, supplementation with SCFP, and especially the SCFPb-2X treatment, attenuated adverse effects of grain-based SARA on the diversity and predicted functionality of microbiota in solid rumen digesta.

Key words: Postbiotics, *Saccharomyces cerevisiae* fermentation products (SCFP), SARA, rumen solid microbiota

5.2 Introduction

High-grain diets are widely used in high-yielding dairy cows to meet their high energy requirements. These diets can result in accumulation of volatile fatty acids (VFA) from rumen fermentation and reduced rumen buffering, resulting in rumen pH depressions (Plaizier et al., 2012). When the rumen pH is depressed for extended periods, such as for more than 180 min/d below 5.6, this can result in subacute ruminal acidosis (SARA) (Cooper et al., 1997; Gozho et al., 2005; Plaizier et al., 2008). Rumen pH below 5.0 represents acute ruminal acidosis and may also involve the accumulation of lactate acid in the rumen (Zebeli et al., 2012; Plaizier et al., 2018; Plaizier et al., 2022). SARA is especially prevalent during early and mid-lactation, due to switches from high forage to high grain diets (Kleen et al., 2003; Plaizier et al., 2008; Valente et al., 2017). A depressed rumen pH can interrupt the barrier function of the rumen epithelium. This disruption causes the translocation of immunogenic compounds such as lipopolysaccharides (LPS), that are released by gram-negative ruminal bacteria, from the rumen into the systemic circulation (Khafipour et al., 2009a; Plaizier et al., 2022). Rumen bacteria produce a wide array of enzymes to break down structural and non-structural carbohydrates, protein and peptides, and triglycerides into absorbable substrates that can then be utilized by the animals (Russell and Rychlik, 2001; Firkins and Yu, 2015; Plaizier et al., 2018; Gruninger et al., 2019).

The composition and functionality of the rumen microbiota can be changed when the composition of the diet changes and the availabilities of substrates for microbiota change, since some microbes can take advantage of the new available substrates while others cannot (Khafipour et al., 2009c; Mao et al., 2013; Agler et al., 2016). The colonization of several microbes in the host relies on other members in the community, such that their competitive advantage can increase when others provide them metabolites. Thus, direct interactions among microbes play an important role in determining the microbiome structures (Faust and Raes, 2012; Agler et al., 2016; Manirajan et al., 2018). To predict the microbe-microbe interactions from repeated measurements of their presence or abundance, co-occurrence analysis is commonly used as microbial network inference (Faust and Raes, 2016). From microbial interactions, “hub taxa” are observed as the most interactive OTUs/ASVs in a microbial network and they are important in shaping the microbiome structures. These hub taxa control the abundances of many other microorganisms as they can suppress the growth and diversity of those microbes (Faust and Raes, 2012; Agler et al., 2016; Manirajan et al., 2018; Derakhshani et al., 2020). Therefore, observing the changes of the interactive microbial connections and hub taxa aids in better understanding the effect of the diet or host phenotype on the entire microbial community. The population of several taxa, such as fibrolytic bacteria that are sensitive to a low pH, decreases when the rumen pH is depressed (Nagaraja and Titgemeyer, 2007; Zhou et al., 2015). Thus, SARA affects the composition and functionality of the rumen microbiota. It has been reported that SARA reduced the richness and evenness of the rumen microbial community, and also the relative abundance of several pH sensitive bacteria, such as fibrolytic bacteria (Mao et al., 2013;

Brede et al., 2020). Although SARA negatively affects milk production, animal health and welfare, its clinical symptoms can be easily unnoticed. Hence, it is critical for the production, health and welfare of dairy cows to prevent SARA and attenuate its adverse effects.

Several strategies have been developed to attenuate the negative effect of SARA, such as supplementing diets with buffers, antibiotics, prebiotics, probiotics and postbiotics. Prebiotics and probiotics are commonly used in recent years as alternatives to antibiotics. Reasons for this, their production include a wide range of antibacterial and bacteriostatic substances, providing a more suitable environment such as available substrates for rumen bacteria including fibrolytic bacteria. As a result, the richness and evenness as well as the robustness of the microbial community are maintained, and animal health and production during SARA are improved (Michalak et al., 2021; Hadhoud et al., 2022). Postbiotics, which are known as functional bioactive compounds, including short-chain fatty acids (SCFAs), functional proteins, metabolites and extracellular polysaccharides, have received more attention in recent years as alternatives (Wegh et al., 2019; Barros et al., 2020). Several studies have shown that supplementation with postbiotics reduces inflammation, promotes milk production and feed efficiency in lactating dairy cows and calves, as well as stabilizes rumen pH and rumen fermentation during high grain feeding (Acharya et al., 2017; Mahmoud et al., 2020; Vailati-Riboni et al., 2021; Guo et al., 2022). Previous studies usually assess the microbia changes only in rumen liquid fraction due to easier sampling methods, few reseaches on rumen solid microbial changes were reported when animals were exposed to metabolic stressors (Martin et al., 2001; Michalet-Doreau et al., 2001; Petri et al., 2013; McCann et al., 2016;

Ji et al., 2017; Brede et al., 2020). It is necessary to investigate the responses of rumen microbiota in both rumen liquid and solid fractions, because the composition and functionality of the microbiota are different in these two fractions so that they may respond differently to stressors and supplemets. This study aimed to evaluate the effects of two postbiotics from *Saccharomyces cerevisiae* fermentation (SCFP) (Original XPC and NutriTek, Diamond V, Cedar Rapids, IA) and their dose on composition, functionality, and interaction among microbes in rumen solid digesta in lactating dairy cows during repeated grain-based SARA challenges.

5.3 Materials and Methods

The protocol used in this study was approved by the University of Manitoba Animal Care Committee (Protocol # F14-038) and followed the guidelines of the Canadian Council for Animal Care (CCAC, 1993).

5.3.1 Animals, diet and experimental design

Complete details of the animal management, diet composition and experimental design are the same as described in Chapter 3 and 4.

Sample collection. Rumen digesta samples were taken and rumen solid digesta were separated as described in Chapter 4. Approximately, 8 g rumen solid sample was aliquoted into plastic bags and snap frozen immediately in liquid nitrogen and stored at -80°C for further microbial analysis.

DNA extraction. Genomic DNA of rumen solid digesta was extracted similarly to that of rumen liquid digesta samples as described in Chapter 4. Rumen solid DNA was

extracted using *Quick-DNA*TM ZR Fecal/Soil DNA kits (D6010; Zymo Research Corp., Orange, CA) following the manufacturer's procedures. These included a 2 min bead-beating step at 1750 strokes per minute using Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ, USA)). Genomic DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and was normalized to 20 ng/μl followed by MiSeq Illumina sequencing. The DNA samples were quality checked by gel electrophoresis and PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al (2009c). Amplicons were verified by agarose gel electrophoresis.

PCR amplification and construction of sequencing libraries. These procedures were the same as described in Chapter 4. The PCR was targeted to amplify the V3-V4 regions of the bacterial 16S rRNA genes using modified F338/R306 primers. The PCR reactions included an initial denaturing step at 94°C for 3 min, followed by 32 amplification cycles at 94°C for 30 s, 55°C for 20 s, and 72°C for 20 s, with a final extension step at 72°C for 5 min in an Eppendorf Mastercycler pro S (Eppendorf, Hamburg, Germany). Subsequently, the sequencing library was generated and sequenced using a MiSeq Reagent Kit V3 (600-cycle; Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada.

5.3.2 Statistical analysis

Bioinformatics and statistical analyses of microbiome data. Bioinformatics and statistical analysis were performed as described in Chapter 4. Briefly, the DADA2 algorithm was used for quality control and the feature table was constructed. Sequences were assigned into amplicon sequence variants (ASVs) and taxonomy was classified with QIIME 2 2023.2 (Bolyen et al., 2019). Only samples with sequencing depth greater than 6072 were kept. Community α -diversity (Chao1, Shannon's diversity index, Observed Features index, Faith's Phylogenetic Diversity (PD) index and Pielou's Evenness index) and β -diversity (Jaccard distance, Bray-Curtis distance, unweighted UniFrac distance and weighted UniFrac distance indices) metrics were computed using QIIME 2 default scripts, at an even depth per sample. Non-metric multidimensional scaling (nMDS) was applied on the resulting Bray-Curtis distance matrices to generate two-dimensional plots using default settings of the PRIMER-E software ver. 7.0.17 (Clarke and Gorley, 2015).

Similar as described in Chapter 4, the UNIVARIATE procedure of SAS (v 9.4, SAS Institute Inc., Cary, NC, USA) was used for testing the normality of residuals for α -diversity and the relative abundances of bacteria analyses. If the residuals were not normal, the data was log or Box-Cox transformed for normalization before subjected to MIXED procedure of SAS as described in the results tables. Block was considered as random effect, stage was repeated measure, parity and treatment were fix effects. The effect of parity was removed from the model when its P-value was greater than 0.10. All pairwise comparisons between the groups were tested using the Tukey studentized range adjustment. Contrast comparisons were made between control and all of the SCFP, SCFPa and SCFPb, and SCFPb-1X and SCFPb-2X. Significant effects were considered

at $P < 0.05$ and tendencies at $0.05 \leq P < 0.1$ were discussed. Data were presented as means from original data in this study.

Permutational multivariate analysis of variance (PERMANOVA; implemented in PRIMER-E software (v.7.0.17)) (Clarke and Gorley, 2015) was used to detect significant differences between β -diversity metrics of bacterial communities. Non-metric multidimensional scaling (nMDS) was applied on the resulting distance matrices to generate two-dimensional plots using default settings of the PRIMER-E. To support the results of PERMANOVA, PERMDISP analysis in PRIMER-E was used to detect the effect of the dispersions on β -diversity of the rumen solid microbiota.

Compositional dynamics of solid digesta bacterial communities of the rumen were assessed using the Metagenomic Longitudinal Differential Abundance (MetaLonDA) method with edgeR package in R (Metwally et al., 2018), similarly as for rumen liquid microbiota (Guo et al., 2023). Taxonomic profiles were normalized with cumulative sum scaling (CSS), and pairwise comparisons were conducted between Control vs. SCFPb-2X, Control vs. SCFPb-1X, and Control vs. SCFPa treatments. Within each comparison, the longitudinal profiles were fitted with a negative binomial smoothing spline. Significant time intervals were identified when P-values < 0.05 after multiple testing corrections by Benjamini-Hochberg (Benjamini and Hochberg, 1995). Data were presented both at the phylum and genus levels.

Functional prediction of rumen solid microbiota. Predicted functions of rumen solid microbiome including Amino Acid, Lipid and Carbohydrates metabolic pathways were assessed by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (CowPI) (Wilkinson et al., 2018). The differences between Control and

SCFP supplementation treatment groups during SARA challenges were analyzed by STAMP v2.1.3 (Parks et al., 2014) with ANOVA test. Significant differences were adjusted using Tukey-Kramer test and then were identified when P-values < 0.05 after multiple testing corrections by Benjamini-Hochberg's false discovery rate (FDR) correction.

Co-occurrence analysis. As described by Guo et al. (2023), correlation network analysis (CoNet) (Derakhshani et al., 2018; Zhang et al., 2018) was used to determine microbial co-occurrence/mutual-exclusion relationships among microbial members under SARA (SARA1 and SARA2) and non-SARA (pre-SARA1, post-SARA1, post-SARA2) conditions. To determine the influential capacity of bacterial taxa (Trosvik and de Muinck, 2015), their degree of connectedness was calculated with dividing the total number of edges (connections) observed for each phylum by its relative abundance in the community. Hub ASVs in each niche were identified when they had more than 15 edges with other members in the community. The correlation between hub ASVs and biodiversity metrics, rumen VFAs and ammonia concentrations was conducted by Spearman's rank correlation coefficient (ρ) and Corrplot package of R (Wei et al., 2017).

The NetCoMi (Network Construction and comparison for Microbiome data) was used to determine if associations between paired microbes and the overall network structure differed among treatments (Peschel et al., 2021). The same data filtering and normalization was used as in CoNet, and, subsequently, group comparisons were conducted by calculating network summary characteristics, including the number of components, clustering coefficients, modularity, positive edge percentage, edge density

and natural connectivity. Network comparison module assessed the overall network structure differences among different treatment groups and experimental periods, using permutation tests with 1,000 replicates, and the differential associations with an alpha of 0.05 were selected (Peschel et al., 2021).

5.4 Results

Three companion papers reported the effects of *Saccharomyces cerevisiae* fermentation products (SCFP) and subacute ruminal acidosis (SARA) on feed intake, milk production, rumen fermentation and nutrient digestibilities (Khalouei et al., 2020), and on bacterial endotoxin concentrations and markers of inflammation (Guo et al., 2022), as well as on the composition and functionality of microbiota in rumen liquid digesta (Guo et al., 2023) in lactating dairy cows.

5.4.1 Alpha- and beta-diversity dynamics of rumen Solid microbiota

The SARA challenges significantly reduced the richness and diversity of microbiota in solid rumen digesta ($P < 0.001$, Table 5-1). There was no interaction effect of treatment and stage on alpha-diversity of these microbiota ($P > 0.1$). The decrease of Chao1 index was more severe during SARA1/2 and SARA2/2 compared with in other stages in SCFPa treatment, and that was more severe during SARA2/2 stage in the Control treatment, while more severe during the SARA1/2 stage in SCFPb-2X treatment ($P < 0.05$). The decrease of the Chao1 index in SCFPb-2X treatment during the SARA2 stage was not significant compared with non-SARA stages. Treatments tended to affect evenness indices of the microbiota in solid rumen digesta ($P = 0.07$). The SCFPa

treatment had lower evenness compared with the Control treatment ($P = 0.05$), whereas no differences were observed between the Control and the other SCFP treatments across experimental stages. No treatment effect was observed on Shannon's diversity, Faith's PD and observed features of the microbiota of rumen solid digesta ($P > 0.1$). The PERMANOVA analysis showed that both SCFP treatment ($P = 0.0001$, Figure 5-1A) and SARA challenges ($P = 0.0001$, Figure 5-1B) affected the β -diversity of microbiota in rumen solid digesta. However, the PERMDISP analysis showed a significant treatment effect ($P = 0.004$, Figure 5-1A), but not a significant SARA challenge effect ($P = 0.07$, Figure 5-1B). The composition of the rumen solid microbiota differed among treatment groups across the SARA stages (Figure 5-1A).

5.4.2 Compositional dynamics of rumen solid microbiota

In total, 14 phyla and 445 genera were identified in the microbiota of rumen solid digesta. The most abundant bacterial phyla in rumen solid microbiota were Firmicutes (77~80%), Bacteroidetes (16~19%), Actinobacteria (0.8~1.1%), Proteobacteria (0.5~0.8%) and Fibrobacteres (0.1~0.3%). The average relative abundances of these phyla during the non-SARA and SARA stages in each treatment group were summarized in Table 5-2. There was a treatment effect on the relative abundance of Fibrobacteres across experimental stages ($P < 0.01$), and the relative abundance of this phylum was lower in the SCFPb-1X treatment compared with the Control and SCFPb-2X treatments ($P < 0.01$). No treatment effect was observed on the relative abundances of other phyla ($P > 0.05$). The SARA stage effect was significant for all phyla ($P < 0.05$). There were no interaction effects of treatment and SARA stage on the relative abundance of any of the

identified phyla ($P > 0.1$). The relative abundances of Actinobacteria was higher in the SARA2 than in the post-SARA2 stage (1.15 vs 0.84%, $P < 0.05$). During the SARA2 stage, the relative abundance of Proteobacteria was higher than during the non-SARA stages ($P < 0.05$) while SARA1 tended to increase compared with post-SARA2 (0.71 vs 0.46%, $P = 0.07$). Meanwhile, both grain-based SARA challenges reduced the relative abundance of Fibrobacteres ($P < 0.001$). Neither SARA stage or SCFP treatment affected the relative abundances of Firmicutes and Bacteroidetes. There was also no treatment or SARA stage effect on the ratio between Firmicutes and Bacteroidetes (F:B, $P > 0.1$).

Comparisons of compositional shifts in the relative abundances of Firmicutes and Bacteroidetes between the Control and SCFP supplementary treatment groups are shown in Figure 5-2. No significant differences in the fitting splines of Firmicutes and Bacteroidetes were observed between the Control and SCFP treatment groups during the SARA challenges. However, Proteobacteria were more abundant in both SCFPb-1X and SCFPb-2X treatments groups compared with the Control treatment group during the first week after parturition ($P < 0.05$, 5-3a and 5-3b). Fibrobacteres was less abundant in the SCFPb-1X treatment group than in the Control treatment group during first week after parturition and during the second SARA challenge (SARA2, $P < 0.05$, Figure 5-3c).

At the genus level, taxa that were significantly more abundant within each SARA stage are shown in Figures 5-4, 5-5, 5-6. Figure 5-4 presents taxa whose relative abundances were increased by the Control and SCFPb-2X treatments across SARA stages. Overall, 47 taxa were increased by the Control or SCFPb-2X treatments during each SARA stage. Briefly, the SCFPb-2X treatment increased the relative abundances of several genera, including members of *Ruminiclostridium*, *Lachnospiraceae* UCG-009,

Candidatus Hepatincola and *Lachnoclostridium* during the first SARA challenge (SARA1). The SCFPb-2X treatment also increased members of family *Bacteroidales* S24-7 group, *Treponema*, *Lactobacillus* and family *Peptococcaceae* during SARA2 ($P < 0.05$). When comparing the Control and SCFPb-1X treatments, the relative abundances of 61 bacterial taxa were increased by one of these two treatment groups within each SARA stage (Figure 5-5). Briefly, the SCFPb-1X treatment increased the relative abundance of *Sharpea* during the first SARA challenge (SARA1), as well as members of *Erysipelotrichaceae* UCG-009, *Megasphaera*, *Lachospiraceae* (*Syntrophococcus*) compared to the Control during SARA2 ($P < 0.05$). A comparison of the Control and SCFPa treatments revealed that the relative abundances of 56 bacterial taxa were increased by one of these two treatments with different colors in the Figure 5-6 representing the taxa that were enriched in the corresponding groups. Briefly, the SCFPa treatment increased the relative abundances of *Prevotella*, *Butyrivibrio* and *Anaerovibrio* during the SARA1 stage, and increased these abundances of members of *Ruminococcaceae*, *Succinivibrionaceae*, *Syntrophococcus*, *Olsenella* and *Lactobacillus* during the SARA2 stage, as well as *Corynebacterium*, *[Eubacterium] ruminantium* and *Streptococcus* during the first week after calving ($P < 0.05$).

Table 5-1. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (from 4 wk before until 12 wk after parturition) on alpha-diversity of rumen solid microbiome in lactating dairy cows

Item	Treatment ¹	Stage ²												SEM	P-value ³		
		wk -4	wk -1	wk 1	wk 3	wk 4 Pre-SARA1	wk 5 SARA1/1	wk 5 SARA1/2	wk 7 Post-SARA1	wk 8 SARA2/1	wk 8 SARA2/2	wk 10 Post-SARA2	wk 12 Post-SARA2		Treat	Stage	Int ⁴
Chao1	Control	598.69 ^{ab}	643.87 ^{ab}	572.01 ^{ab}	637.97 ^{ab}	637.24 ^{ab}	471.74 ^{ab}	436.11 ^{ab}	633.58 ^{ab}	468.06 ^{ab}	358.43 ^b	658.87 ^a	592.65 ^{ab}	29.58	0.86	<.0001	0.52
	SCFPa	584.39 ^{abc}	631.54 ^{abc}	569.51 ^{abc}	716.41 ^{ab}	736.55 ^a	453.00 ^{abc}	390.30 ^c	656.60 ^{ab}	370.94 ^c	352.13 ^c	662.37 ^{ab}	637.68 ^{ab}	30.11			
	SCFPb-1X	709.41 ^a	597.04 ^{ab}	675.93 ^{ab}	618.30 ^{ab}	603.95 ^{abA}	399.69 ^{bB}	379.66 ^b	656.30 ^a	382.65 ^b	318.27 ^b	675.62 ^a	551.95 ^{ab}	28.65			
	SCFPb-2X	580.63 ^{ab}	568.59 ^{ab}	635.18 ^{ab}	493.86 ^{ab}	763.13 ^{aA}	448.54 ^{abB}	385.30 ^b	606.32 ^{ab}	453.84 ^{ab}	467.84 ^{ab}	594.84 ^{ab}	672.05 ^{ab}	31.96			
Shannon's diversity	Control	8.56 ^a	8.49 ^a	8.35 ^{acd}	8.57 ^a	8.59 ^a	7.76 ^{cde}	7.63 ^c	8.46 ^a	7.71 ^{de}	7.22 ^c	8.61 ^a	8.44 ^{ac}	0.12	0.75	<.0001	0.71
	SCFPa	8.45 ^b	8.59 ^b	8.01 ^b	8.67 ^{ab}	8.78 ^a	7.21 ^c	7.34 ^c	8.65 ^b	6.84 ^c	6.98 ^c	8.53 ^b	8.39 ^b	0.12			
	SCFPb-1X	8.74 ^a	8.43 ^{ab}	8.65 ^{ab}	8.29 ^b	8.41 ^{ab}	6.90 ^c	7.24 ^c	8.46 ^{ab}	7.03 ^c	7.02 ^c	8.51 ^{ab}	8.34 ^{ab}	0.12			
	SCFPb-2X	8.4339 ^a	8.52 ^a	8.63 ^a	8.36 ^{ab}	8.76 ^a	7.70 ^{bc}	7.37 ^c	8.59 ^a	7.55 ^{bc}	7.42 ^c	8.56 ^a	8.69 ^a	0.13			
Pielou's Evenness	Control	0.93 ^a	0.91 ^a	0.91 ^a	0.93 ^a	0.92 ^a	0.88 ^b	0.87 ^b	0.91 ^a	0.87 ^b	0.85 ^b	0.92 ^a	0.92 ^a	0.01	0.07	<.0001	0.27
	SCFPa	0.92 ^{ab}	0.93 ^a	0.87 ^b	0.93 ^a	0.93 ^a	0.82 ^c	0.85 ^c	0.93 ^a	0.81 ^c	0.83 ^c	0.91 ^{ab}	0.90 ^{ab}	0.01			
	SCFPb-1X	0.93 ^a	0.92 ^{ab}	0.92 ^{ab}	0.90 ^b	0.91 ^{ab}	0.81 ^c	0.85 ^c	0.91 ^b	0.82 ^c	0.85 ^c	0.91 ^{ab}	0.91 ^{ab}	0.01			
	SCFPb-2X	0.92 ^a	0.93 ^a	0.93 ^a	0.94 ^a	0.92 ^a	0.88 ^b	0.86 ^b	0.93 ^a	0.86 ^b	0.84 ^b	0.93 ^a	0.93 ^a	0.01			
Faith's Phylogenetic diversity	Control	98.38 ^a	101.96 ^a	92.88 ^{ab}	105.43 ^a	102.99 ^a	77.93 ^{bc}	71.73 ^c	97.73 ^a	77.79 ^{bc}	64.65 ^c	99.68 ^a	98.50 ^a	4.09	0.92	<.0001	0.50
	SCFPa	95.16 ^a	97.02 ^a	95.23 ^a	106.64 ^a	108.52 ^a	71.20 ^b	67.72 ^b	100.68 ^a	62.27 ^b	65.63 ^b	101.80 ^a	100.40 ^a	4.12			
	SCFPb-1X	108.85 ^a	100.02 ^{ab}	105.91 ^{ab}	99.32 ^{ab}	99.58 ^{ab}	66.64 ^c	67.00 ^c	102.86 ^{ab}	66.92 ^c	57.03 ^c	101.38 ^{ab}	91.99 ^b	4.10			
	SCFPb-2X	97.77 ^b	98.93 ^b	103.13 ^{ab}	90.45 ^{bc}	115.24 ^a	76.36 ^c	69.67 ^c	98.81 ^b	77.19 ^c	77.01 ^c	99.88 ^{ab}	104.46 ^{ab}	4.42			
Observed features	Control	604.25 ^a	644.09 ^a	579.75 ^{ab}	632.93 ^a	633.00 ^a	479.50 ^{bc}	439.37 ^{bc}	624.25 ^a	472.37 ^{bc}	382.12 ^c	646.25 ^a	599.50 ^a	34.60	0.97	<.0001	0.55
	SCFPa	590.98 ^a	623.65 ^a	576.25 ^{ab}	635.93 ^a	742.62 ^a	440.88 ^{bc}	406.37 ^c	647.50 ^a	366.62 ^c	376.25 ^c	639.00 ^a	633.75 ^a	34.96			
	SCFPb-1X	694.37 ^a	598.12 ^a	687.12 ^a	616.42 ^a	594.50 ^a	381.25 ^b	396.87 ^b	644.00 ^a	386.82 ^b	329.77 ^b	666.91 ^a	562.87 ^a	34.75			
	SCFPb-2X	586.86 ^{bc}	576.86 ^{bc}	627.86 ^{ab}	481.13 ^{bcd}	751.86 ^a	453.57 ^d	407.29 ^d	619.57 ^{ab}	455.86 ^{cd}	464.14 ^{cd}	600.29 ^b	660.67 ^{ab}	37.45			

^{a-d}Means in a row with different superscripts among Stages are different ($P < 0.05$).

^{A-B}Means in a row with different superscripts among Stages tended to be different ($0.05 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during wk 5 (SARA1) and wk 8 (SARA2) after parturition. Rumen samples were taken on d 2 (SARA1/1, SARA1/2) and d 5 (SARA2/1, SARA2/2) during each SARA week. Wk 4 was considered as Pre-SARA1, wk 7 as Post-SARA1, and wk 10 and 12 as Post-SARA2.

³Statistical analyses were conducted on log10-transformed data for Chao1, original data for Faith's Phylogenetic diversity, Box-cox-transformed data for the rest. Presented means are original values prior to transformation.

⁴Int: Interaction effect of treatment and stage.

Table 5-2. Effects of treatment (Control, SCFPa, SCFPb-1X, and SCFPb-2X), stage of subacute ruminal acidosis (SARA) induction (Pre-SARA1, SARA1, Post-SARA1, SARA2, and Post-SARA2) on the relative abundances (%) of the main phylum of rumen solid microbiome in lactating dairy cows

Item	Treatment ¹				Stage ²						P- values ³					
	Control	SCFPa	SCFPb-1X	SCFPb-2X	Pre-SARA1	SARA1	Post-SARA1 (wk 7)	SARA2	Post-SARA2 (wk 10)	SEM	Treat	Stage	Treat × Stage	Contrasts ⁴		
														1	2	3
Actinobacteria	0.93	1.06	1.02	0.85	0.90 ^{ab}	1.09 ^{ab}	0.86 ^{ab}	1.15 ^a	0.84 ^b	0.08	0.33	0.04	0.11	0.65	0.23	0.17
Bacteroidetes	17.26	16.56	16.82	18.39	15.79	18.60	17.22	18.23	16.44	0.86	0.53	0.21	0.48	0.99	0.34	0.23
Firmicutes	78.78	79.39	79.21	77.91	80.28	77.72	78.92	77.39	79.82	0.96	0.73	0.21	0.55	0.96	0.49	0.36
Proteobacteria	0.54	0.59	0.72	0.54	0.49 ^b	0.71 ^{abA}	0.51 ^b	0.89 ^a	0.46 ^{bB}	0.07	0.31	<0.001	0.83	0.40	0.70	0.10
Fibrobacteres	0.26 ^a	0.23 ^{ab}	0.16 ^b	0.27 ^a	0.44 ^a	0.10 ^b	0.31 ^a	0.09 ^b	0.38 ^a	0.04	0.006	<0.001	0.53	<0.001	0.36	0.01
F:B ⁵	4.59	4.87	4.75	4.25	5.11	4.26	4.59	4.28	4.87	0.28	0.53	0.33	0.52	0.60	0.24	0.42

^{a-b}Means in a row with different superscripts among Treatments are different ($P < 0.05$).

^{A,B}Means in a row with different superscripts among Treatments tended to be different ($0.05 \leq P < 0.1$).

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during weeks 5 (SARA1) and 8 (SARA2) post-calving. Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 as Post-SARA2.

³Statistical analyses were conducted on log-transformed data for Actinobacteria and Proteobacteria, original data for Bacteroidetes and Firmicutes, and Box-cox data for Fibrobacteres and F:B. Presented means are original values prior to transformation.

⁴Contrasts: 1 = Control vs. SCFP, 2 = SCFPa vs. SCFPb, 3 = SCFPb-1X vs. SCFPb-2X.

⁵F:B: Ratio of Firmicutes: Bacteroidetes.

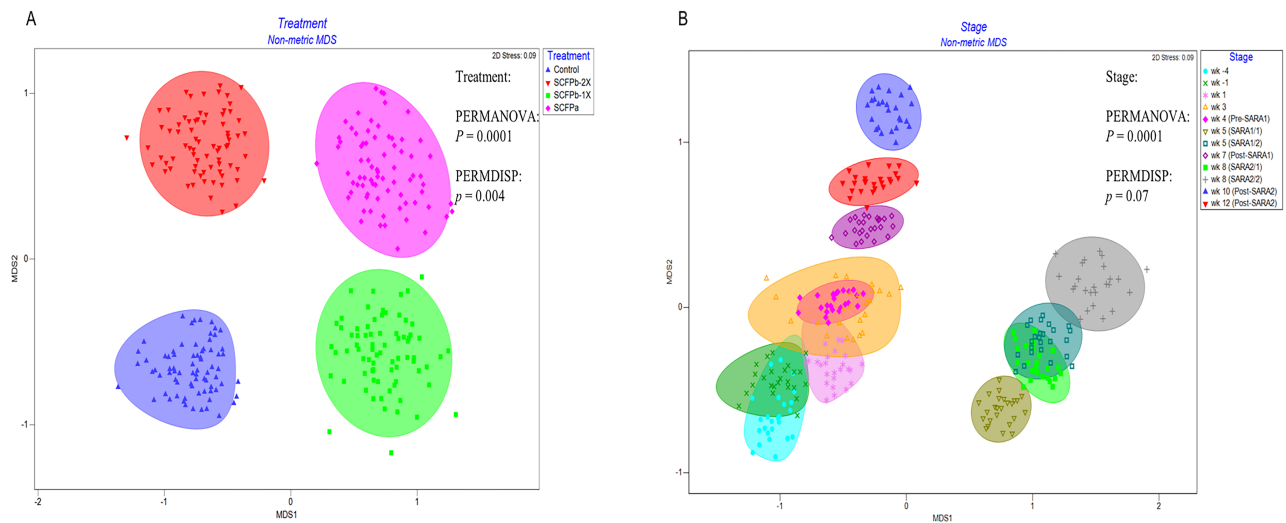


Figure 5-1. nMDS of Bray-Curtis distances of rumen solid microbial communities among treatments (A) and stages (B). The ASV table was normalized using cumulative sum scaling (CSS) transformation. Permutational multivariate analysis of variance (PERMANOVA) was used to detect the distinction of clustering patterns between treatments and stages. PERMDISP analysis in Primer 7 was used to detect the effect of the dispersions on β -diversity of the rumen liquid microbiota. $P < 0.05$ was considered as significant differences. The effect of block was considered as random factor in all comparison. The experimental stage started from 4 weeks before until 12 weeks after calving. SARA challenges were conducted on wk 5 and wk 8 after parturition. Rumen samples were taken weekly but twice during SARA weeks (SARA1/1, SARA1/2, SARA2/1, SARA2/2). Week 4 was considered as Pre-SARA1, week 7 as Post-SARA1, and week 10 and 12 as Post-SARA2.

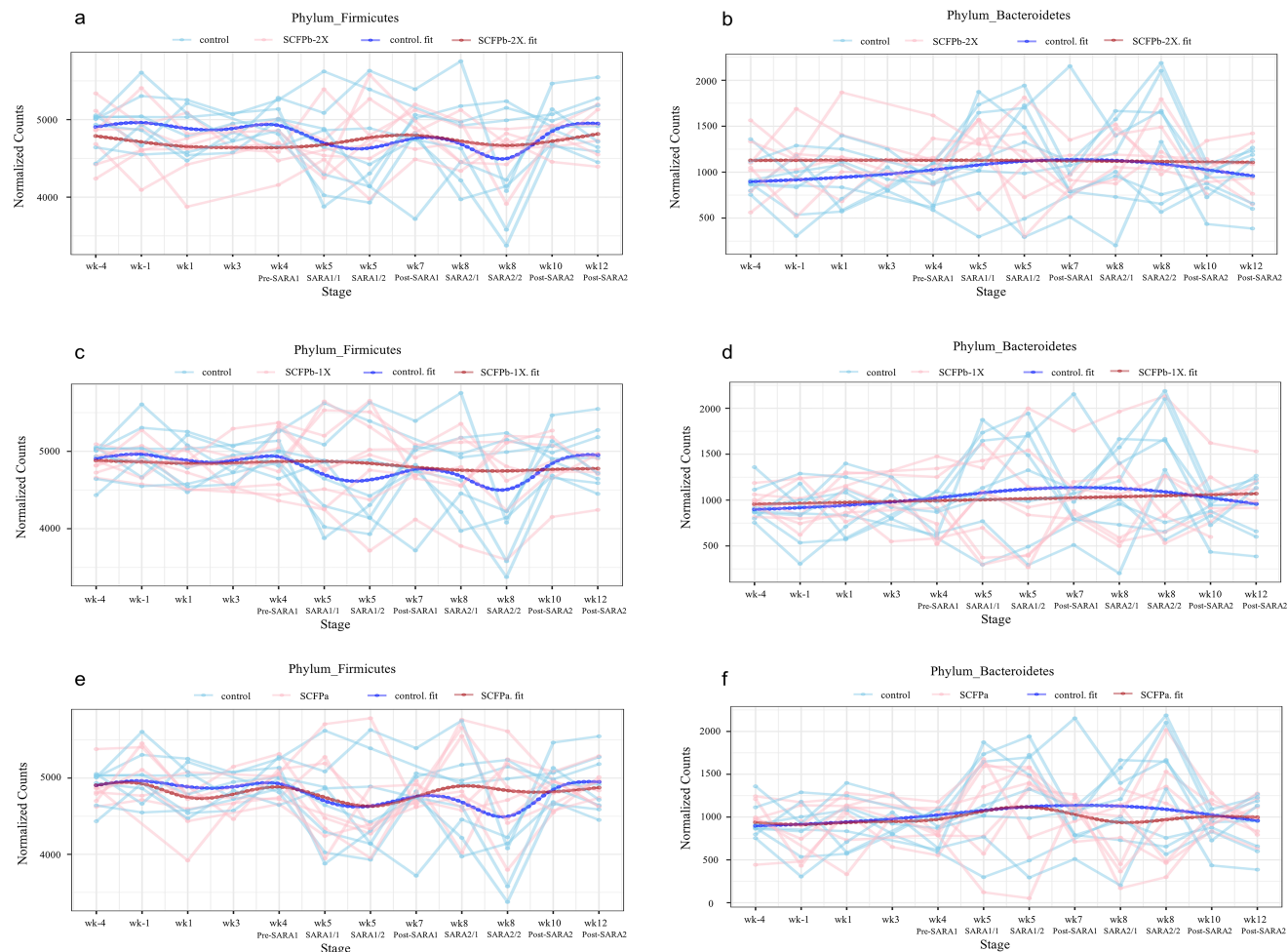


Figure 5-2. Longitudinal shifts in Firmicutes and Bacteroidetes proportions in rumen solid microbiota. Metagenomic Longitudinal Differential Abundance (MetaLonDA) was used to test the longitudinal changes in rumen solid microbiota at the phylum level as lactation progressed. The ASV table was normalized using cumulative sum scaling (CSS) transformation. The longitudinal profiles in each group were fitted with a negative binomial smoothing spline. Blue color represents control and red represents SCFP groups. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. a, b) Longitudinal shifts in Firmicutes and Bacteroidetes proportions, respectively, in control vs. SCFPb-2X group. c, d) Longitudinal shifts in Firmicutes and Bacteroidetes proportions, respectively, in control vs. SCFPb-1X group. e, f) Longitudinal shifts in Firmicutes and Bacteroidetes proportion, respectively, in control vs. SCFPa group. Cows received treatments from 4 weeks before until 12 weeks after parturition. SARA challenges were conducted on wk 5 and wk 8. Rumen samples were collected weekly but twice during SARA weeks (SARA1/1, SARA1/2, SARA2/1, SARA2/2).

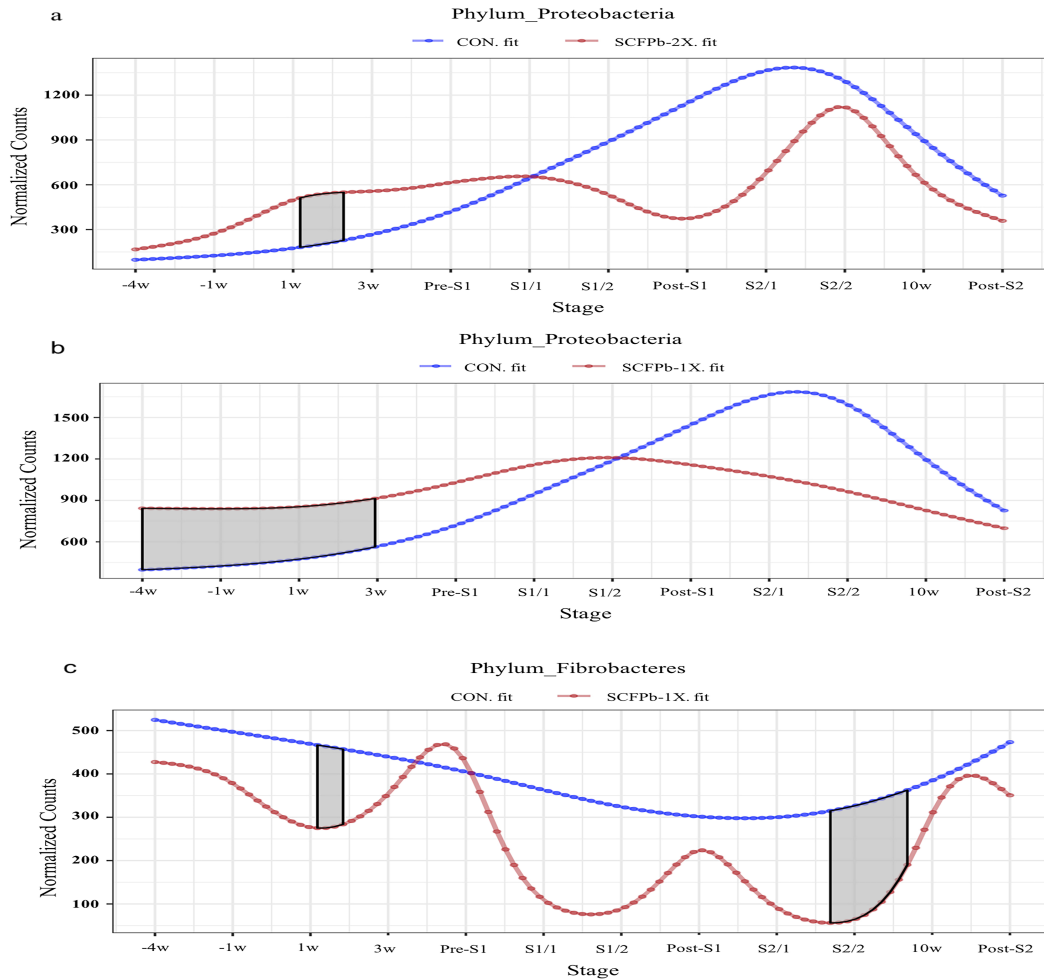


Figure 5-3. Longitudinal shifts in Proteobacteria and Fibrobacteres proportions in rumen solid microbiota. Metagenomic Longitudinal Differential Abundance (MetaLonDA) was used to test the longitudinal changes in rumen solid microbiota at the phylum level as lactation progressed. The ASV table was normalized using cumulative sum scaling (CSS) transformation. The longitudinal profiles in each group were fitted with a negative binomial smoothing spline. Blue color represents control and red represents SCFP groups. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. a) Longitudinal shifts in Proteobacteria proportion in control vs. SCFPb-2X group. b) Longitudinal shifts in Proteobacteria proportion in control vs. SCFPb-1X group. c) Longitudinal shifts in Fibrobacteres proportion in control vs. SCFPb-1X group. The dark area in figures indicates the significant difference of corresponding phylum between two treatment groups ($P < 0.05$). Cows received treatments from 4 weeks before until 12 weeks after parturition. SARA challenges were conducted on wk 5 and wk 8. Rumen samples were collected weekly but twice during SARA weeks (SARA1/1, SARA1/2, SARA2/1, SARA2/2).

5.4.3 Predicted functionality of rumen solid microbiota

The predicted functionality of rumen solid microbiota revealed 254 endogenous third-level KEGG pathways using CowPi. Of these, 150 were considered rumen microbial metabolic pathways. The comparison between the Control and SCFP groups during the SARA challenges is shown Figure 5-7. Compared with Control, SCFP supplementation promoted 3 carbohydrates metabolic pathways including “ascorbate and aldarate metabolism”, “fructose and mannose metabolism”, “inositol phosphate metabolism”, and inhibited 2 carbohydrates metabolic pathways including “starch and sucrose metabolism” and “citrate cycle (TCA cycle)” during SARA challenges. Furthermore, SCFP supplementation inhibited 3 amino acids metabolic pathways including “valine, leucine and isoleucine biosynthesis”, “histidine metabolism”, “phenylalanine metabolism” and promoted 1 amino acid metabolic pathway “amino acid metabolism” compared with the control treatment during SARA challenges. In addition, SCFP supplementation promoted 3 lipid metabolic pathways including “primary bile acid biosynthesis”, “secondary bile acid biosynthesis”, “synthesis and degradation of ketone bodies”, but inhibited 1 lipid metabolic pathway “arachidonic acid metabolism” compared with the Control treatment during the SARA challenges. A comparison of predicted microbial functionalities between the Control treatment and each SCFP treatment groups during SARA showed that 3 carbohydrates pathways, including “ascorbate and aldarate metabolism”, “fructose and mannose metabolism”, “inositol phosphate metabolism”, 1 amino acids “amino acid metabolism” and 3 lipid including “primary bile acid biosynthesis”, “secondary bile acid biosynthesis”, “synthesis and degradation of ketone bodies” metabolic pathways were promoted by the SCFPa

treatment ($P < 0.05$, Figure 5-8). However, no differences in these predicted functionalities were observed between the Control and SCFPb treatment groups (data not shown).

5.4.4 Effect of SCFP supplementation and SARA inductions on co-occurrence patterns of rumen solid microbiota

Figure 5-9 shows that the SARA challenges reduced the number of positive connections among bacterial taxa in the SCFPb treatment group, but not in the Control or SCFPa treatment groups (Figure 5-9a). The SARA challenges also decreased the number of negative connections in the Control and SCFPa treatment groups. No negative connections were detected in the SCFPb treatment group during the SARA challenges (Figure 5-9b). The relative degree of connectedness of each phylum (total number of positive and negative edges observed for each phylum divided by its relative abundance in the community) varied among treatment groups during the SARA stages (Figure 5-9a and 5-9b).

During non-SARA stages, the degrees of positive and negative connections were higher in SCFP treatment groups compared to the Control treatment group, and these connections were higher in the SCFPb treatment group than in the SCFPa treatment group (Figure 5-9a). Bacteroidetes had the highest degree of positive connections in the SCFPb-2X and SCFPa treatment groups. In descending order, Bacteroidetes, Actinobacteria and Proteobacteria showed high degrees of positive connections in the SCFPb-1X treatment groups, whereas Firmicutes and Fibrobacteres showed high degrees of positive connections in the SCFPa treatment group. Meanwhile, Fibrobacteres had the highest degree of positive connections in the SCFPb-1X treatment group, whereas

Firmicutes had the highest degree of connections in the Control treatment group. Fibrobacteres had the highest degree of negative connections in the Control treatment group followed by Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. Bacteroidetes had the highest degree of connections in the SCFPa treatment group, followed by Firmicutes, Proteobacteria and Fibrobacteres. No negative connections were observed in SCFPb treatment groups (Figure 5-9b).

Across SARA challenges, the SCFP treatment groups had higher positive connections than the Control treatment group (Figure 5-9a). Actinobacteria contributed the most to the positive connections in SCFPb treatment groups, followed by Bacteroidetes and Firmicutes. In the SCFPa treatment group, Bacteroidetes had the highest number of positive connections. Bacteroidetes and Firmicutes contributed equally to positive connections in the Control treatment group (Figure 5-9a). Actinobacteria, Bacteroidetes and Firmicutes had equal numbers of negative connections in Control group, whereas in the SCFPa group, Firmicutes was the major contributor to the negative connections, followed by Bacteroidetes and Actinobacteria (Figure 5-9b).

Hub taxa were identified as taxa with a high number (> 15) of positive or negative connections with other members of the community (Figure 5-10). The SARA challenges reduced the number of hub taxa in the Control and SCFPb treatment groups, but not in the SCFPa treatment group. In the Control treatment group, all hub taxa were from the Firmicutes phylum during both SARA and non-SARA stages (Figure 5-10a and 5-10b). During non-SARA stages, cows in Control group had 9 negatively connected hub taxa (3 OTUs from *Lachnospiraceae* NK3A20 group and 1 from family *Lachnospiraceae*, 4 OTUs from *Ruminococcaceae* NK4A214 group and 1 from *Ruminococcus*) and 1

positively connected hub taxon (from genera *Oribacterium*) (Figure 5-10a). During SARA stages, Control cows had 2 negatively connected hub taxa (1 OTU from *Oribacterium* and 1 from *Mitsuokella*) and 1 positively connected taxa from family *Lachnospiraceae* (Figure 5-10b). In the SCFPb-2X treatment group, all positively connected hub taxa were from the Bacteroidetes and Firmicutes phylum during non-SARA stages and from the Bacteroidetes, Firmicutes and Actinobacteria phyla during SARA stages (Figure 5-10c and 5-10d). During non-SARA stages, SCFPb-2X cows had positively connected hub taxa from *Prevotellaceae* (1 OTU from *Prevotellaceae* UCG-003, 5 from *Prevotella*), *Christensenellaceae* (4 OTUs from genera *Christensenellaceae* R-7 group), *Lachnospiraceae* (1 OTU from *Lachnospiraceae* NK3A20 group, 5 from family *Lachnospiraceae*, 1 from *Lachnospiraceae* UCG-006), *Ruminococcaceae* (2 OTUs from *Ruminococcaceae* NK4A214 group, 2 from family *Ruminococcaceae* and 2 from *Saccharofermentans*) and 1 OTU from genera *Rikenellaceae* RC9 gut group (Figure 5-10c). During SARA stages, SCFPb-2X cows had positively connected hub taxa from *Lachnospiraceae* (1 OTU from genera *Lachnospiraceae* NK3A20 group and 2 from family *Lachnospiraceae*), *Ruminococcaceae* (1 OTU from genera *Anaerotruncus*), 1 OTU from family *Prevotellaceae*, family *Bacteroidales* *Incertae Sedis* (1 from genera *Phocaeicola*) and family *Coriobacteriaceae* (1 OTU from genera *Senegalimassilia*) (Figure 5-10d). Similarly, all positively connected hub taxa in the SCFPb-1X treatment group were from the Bacteroidetes and Firmicutes phyla during non-SARA stages and from the Firmicutes phylum during SARA stages (Figure 5-10e and 5-10f).

During the SARA stages, hub taxa were from *Ruminococcaceae* (1 OTU from genera *Anaerotruncus*, 3 from genera *Saccharofermentans*, 1 from family *Ruminococcaceae* and

1 from *Ruminococcaceae* UCG-014), *Christensenellaceae* (5 OTUs from *Christensenellaceae* R-7 group), *Lachnospiraceae* (1 OTU from [*Eubacterium*] *xylanophilum* group, 1 from *Lachnospiraceae* XPB1014 group and 3 from family *Lachnospiraceae*), *Erysipelotrichaceae* (1 OTU from *Erysipelotrichaceae* UCG-004, 1 from *Erysipelotrichaceae* UCG-007, 1 from *Solobacterium*, 1 from [*Anaerorhabdus*] *furcosa* group), *Bacillaceae* (1 OTU from *Bacillus*), *Eubacteriaceae* (1 OTU from *Anaerofustis*), *Rikenellaceae* (3 OTUs from *Rikenellaceae* RC9 gut group), *Prevotellaceae* (1 OTU from *Prevotella*, 1 from *Prevotellaceae* UCG-003, 1 from family *Prevotellaceae*) and 1 OTU from Family XIII AD3011 group (Figure 5-10e). During SARA stages, hub taxa were from *Ruminococcaceae* (1 OTU from *Caproiciproducens*, 1 from *Saccharofermentans* and 2 from family *Ruminococcaceae*) and 2 OTUs from family *Lachnospiraceae* (Figure 5-10f). In the SCFPa treatment group, hub taxa that were negatively connected were from Bacteroidetes and Firmicutes during non-SARA stages and had a combination of positively and negatively connected hub taxa were from Firmicutes during SARA stages (Figure 5-10g and 5-10h).

During non-SARA stages, SCFPa cows had hub taxa from *Lachnospiraceae* (1 OTU from *Lachnospiraceae* XPB1014 group, 1 from [*Ruminococcus*] *gnavreuii* group, 1 from *Lachnospiraceae* NK3A20 group and 2 from family *Lachnospiraceae*), *Ruminococcaceae* (1 OTU from *Ruminococcus*, 1 from *Ruminococcaceae* NK4A214 group, 1 from *Saccharofermentans* and 1 from family *Ruminococcaceae*), *Erysipelotrichaceae* (1 OTU from *Solobacterium* and 1 OTU from *Sharpea*), *Christensenellaceae* (1 OTU from *Christensenellaceae* R-7 group) and *Prevotellaceae* (1 OTU from *Prevotella*) (Figure 5-10g). During SARA challenges, cows had 6 positively

connected hub taxa (1 OTU from *Lachnospiraceae* NK3A20 group, 4 OTUs from genera *Succiniclasticum* and 1 OTU from *Ruminococcaceae* NK4A214 group) and 7 negatively connected taxa (3 OTUs from *[Ruminococcus] gauvreauii* group, 1 OTU from family *Lachnospiraceae*, 1 OTU from family *Bacteroidales* S24-7 group, 1 OTU from *Erysipelotrichaceae* UCG-007 and 1 OTU from *Ruminococcus*) (Figure 5-10h).

As shown in Table 5-3, results from the NetCoMi analysis indicated that network characteristics, including modularity ($P = 0.04$), clustering coefficient ($P = 0.09$), positive edge percentage ($P = 0.07$) and edge density were increased during SARA stages compared to non-SARA stages in the Control treatment group. In the SCFPb-1X treatment group, the clustering coefficient ($P = 0.02$), positive edge percentage ($P < 0.001$), edge density ($P < 0.001$) and natural connectivity ($P < 0.001$) were higher during SARA stages compared to non-SARA stages. In the SCFPa treatment group, the clustering coefficient ($P = 0.02$), positive edge percentage ($P < 0.001$), edge density ($P < 0.001$) and natural connectivity ($P < 0.001$) were also increased, while modularity decreased ($P = 0.04$) during SARA stages compared to non-SARA stages. However, there was no difference of network characteristics between non-SARA and SARA stages in the SCFPb-2X treatment group.

Table 5-3. The comparison of network properties in each treatment group (Control, SCFPa, SCFPb-1X, and SCFPb-2X) between non-SARA and SARA stages.

Treat ¹	Characteristics	Stage ²		Abs.diff.	P-value ³
		Non-SARA	SARA		
Control	Number of components	29	9	20	0.006
	Clustering coefficient	0.35	0.48	0.13	0.09
	Modularity	0.45	0.59	0.14	0.04
	Positive edge percentage	78.76	94.04	15.27	0.07
	Edge density	0.03	0.05	0.01	0.09
	Natural connectivity	0.01	0.02	0.006	0.16
SCFPb-2X	Number of components	19	15	4	0.58
	Clustering coefficient	0.44	0.44	0	0.99
	Modularity	0.44	0.36	0.07	0.36
	Positive edge percentage	81.77	75.60	6.17	0.39
	Edge density	0.04	0.05	0.01	0.21
	Natural connectivity	0.03	0.03	0.001	0.73
SCFPb-1X	Number of components	23	7	16	0.002
	Clustering coefficient	0.43	0.63	0.2	0.02
	Modularity	0.30	0.22	0.07	0.42
	Positive edge percentage	70.58	99.36	28.77	< 0.001
	Edge density	0.05	0.17	0.11	< 0.001
	Natural connectivity	0.03	0.14	0.11	< 0.001
SCFPa	Number of components	24	4	20	0.002
	Clustering coefficient	0.30	0.51	0.21	0.007
	Modularity	0.61	0.46	0.15	0.04
	Positive edge percentage	82.52	100	17.47	< 0.001
	Edge density	0.03	0.12	0.09	< 0.001
	Natural connectivity	0.02	0.07	0.05	< 0.001

¹Treatment: Control = 140 g/d ground corn; SCFPa = 14 g/d Diamond V Original XPC mixed with 126g/d ground corn; SCFPb-1X = 19 g/d NutriTek mixed with 121 g/d ground corn; SCFPb-2X = 38 g/d NutriTek mixed with 102 g/d ground corn.

²Stage: SARA was induced during wk 5 (SARA1) and wk 8 (SARA2) after parturition. Non-SARA stage includes pre-SARA1(wk 4), post-SARA1(wk 7) and post-SARA2 (wk 10 and 12). SARA stage includes SARA1 and SARA2.

³P-value: Significance was considered as $P < 0.05$, and a tendency was considered as $0.05 \leq P < 0.1$.

5.4.5 Correlation between hub taxa, biodiversity metrics of rumen solid microbiota and rumen fermentation characteristics

The association between hub taxa and biodiversity metrics of rumen solid microbiota, rumen VFA and ammonia concentrations that were published in a companion paper (Khalouei et al., 2020) are shown in Figure 5-11. A group of OTUs from Bacteroidetes, including *Rikenellaceae RC9 gut group*, Firmicutes including *Christensenellaceae R-7 group*, *Family XIII AD3011 group*, *Lachnospiraceae XPB1014 group*, unclassified family *Lachnospiraceae*, *Anaerotruncus*, *Ruminococcaceae NK4A214 group*, unclassified family *Ruminococcaceae*, *[Anaerorhabdus] furcosa group*, and genera *Saccharofermentans* were negatively correlated with rumen concentrations of propionate, butyric, valerate and lactate (Figure 5-11a, $P < 0.05$). However, they were positively correlated with rumen concentrations of acetate and ammonia and with α -, β -diversity metrics of the microbiota in solid rumen digesta (Figure 5-11a and 5-11b, $P < 0.05$). A group of OTUs from Firmicutes that included members of the *Ruminococcus gauvreauii* and *Sharpea* genera were positively correlated with rumen propionate and butyrate concentrations but was negatively correlated with rumen acetate and ammonia concentrations and with α -, β -diversity metrics of the rumen solid microbiota (Figure 5-11, $P < 0.05$).

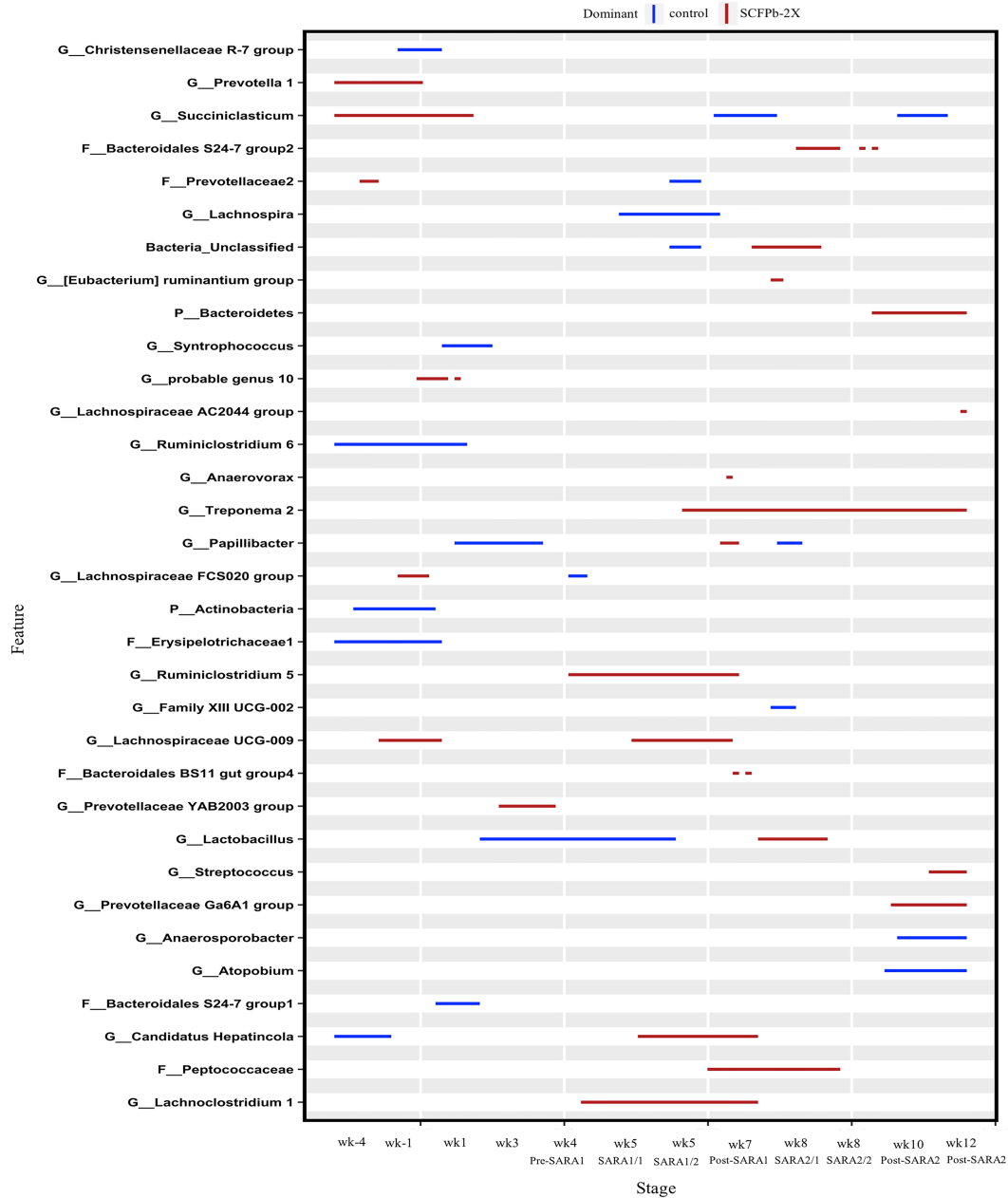


Figure 5-4. Differentially abundant taxa in rumen solid microbiota in control vs. SCFPb-2X. The graph summarizes data that were generated using Metagenomic Longitudinal Differential Abundance (MetaLonDA) method. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. X axes represent stage relative to parturition. Y axes represent taxa that were promoted by control (blue lines) and SCFPb-2X groups (red lines) during corresponding stages.

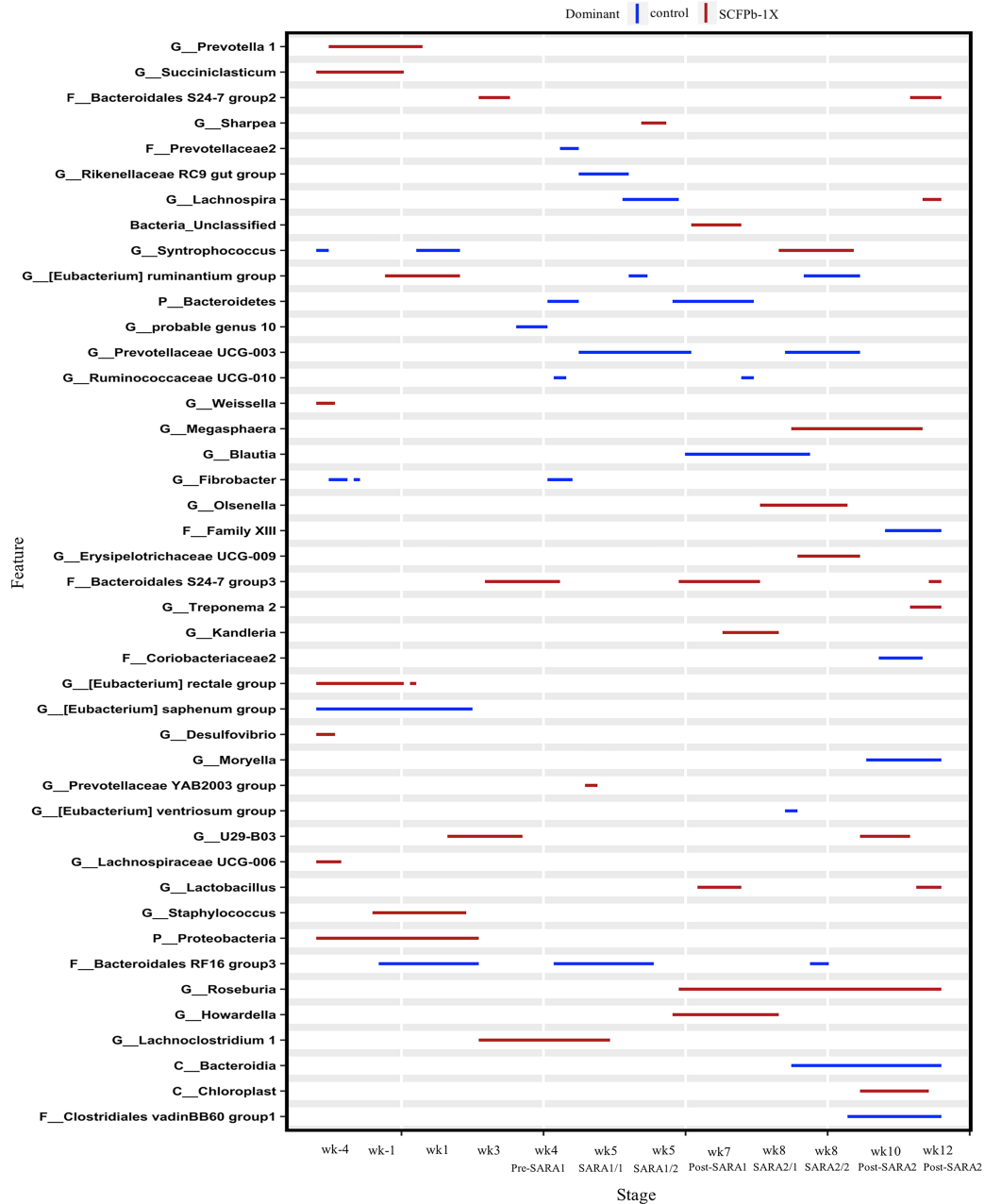


Figure 5-5. Differentially abundant taxa in rumen solid microbiota in control vs. SCFPb-1X. The graph summarizes data that were generated using Metagenomic Longitudinal Differential Abundance (MetaLonDA) method. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. X axes represent stage relative to parturition. Y axes represent taxa that were promoted by control (blue lines) and SCFPb-1X groups (red lines) during corresponding stages.

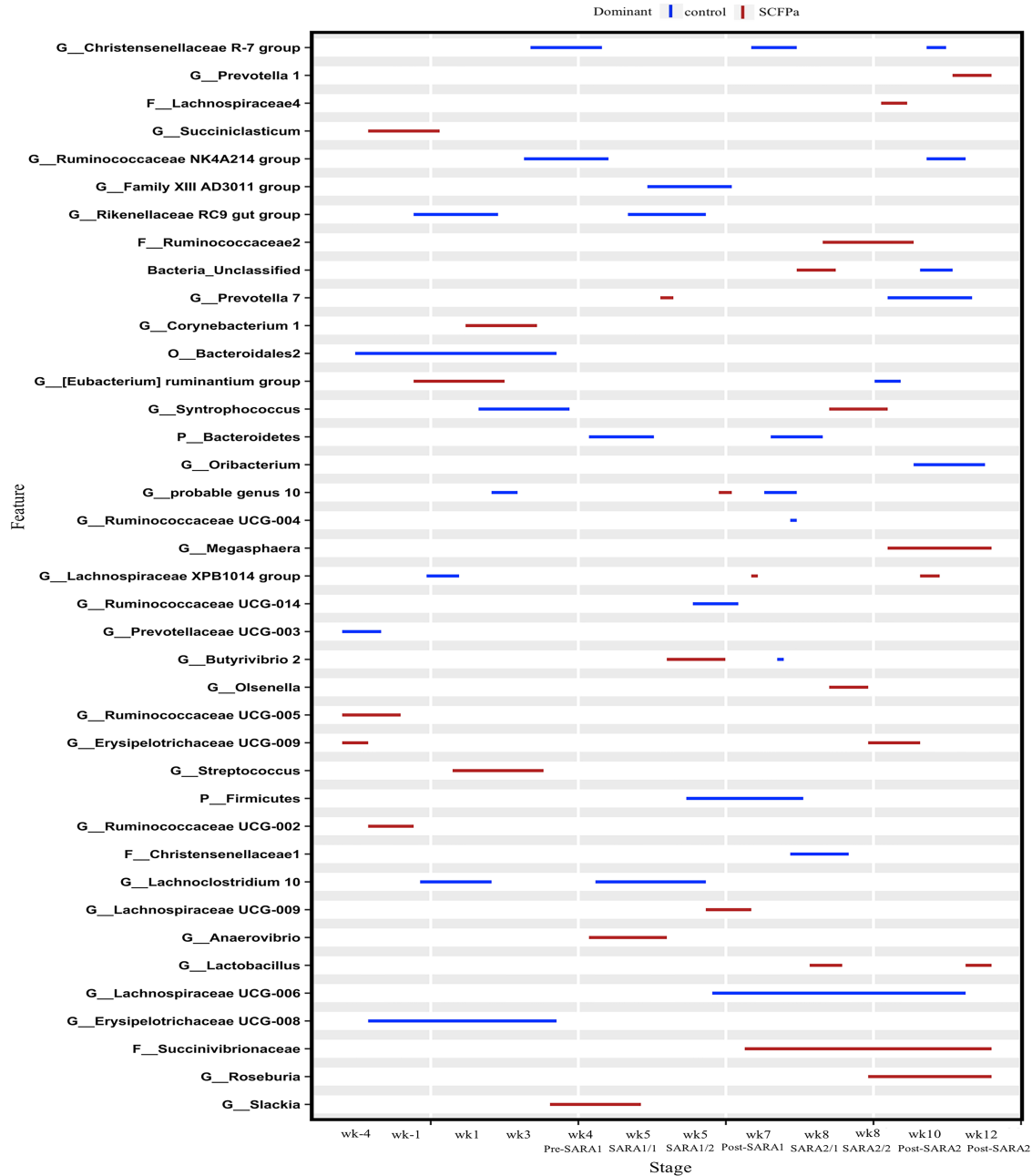


Figure 5-6. Differentially abundant taxa in rumen solid microbiota in control vs. SCFPa.

The graph summarizes data that were generated using Metagenomic Longitudinal Differential Abundance (MetaLonDA) method. The significant time intervals were identified when $P < 0.05$ after multiple testing corrections using Benjamini-Hochberg False Discovery Rate. X axes represent stage relative to parturition. Y axes represent taxa that were promoted by control (blue lines) and SCFPa groups (red lines) during corresponding stages.

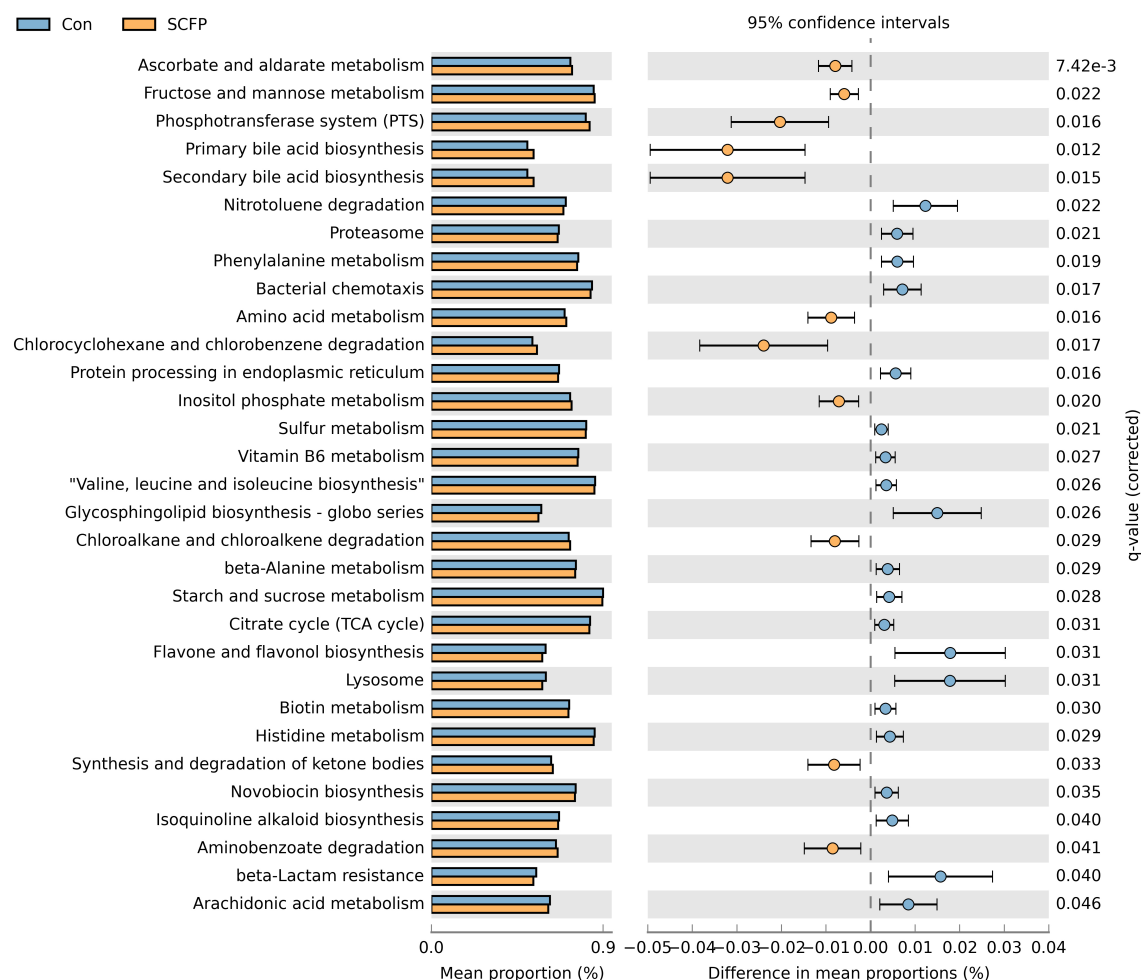


Figure 5-7. Differences in predicted functions of rumen solid microbiota between control and SCFP groups during SARA. Functionalities of rumen solid microbiota were predicted using CowPi. Output was analyzed using STAMP following log_transformation and False Discovery Rate correction. Significant differences were considered as $P < 0.05$.

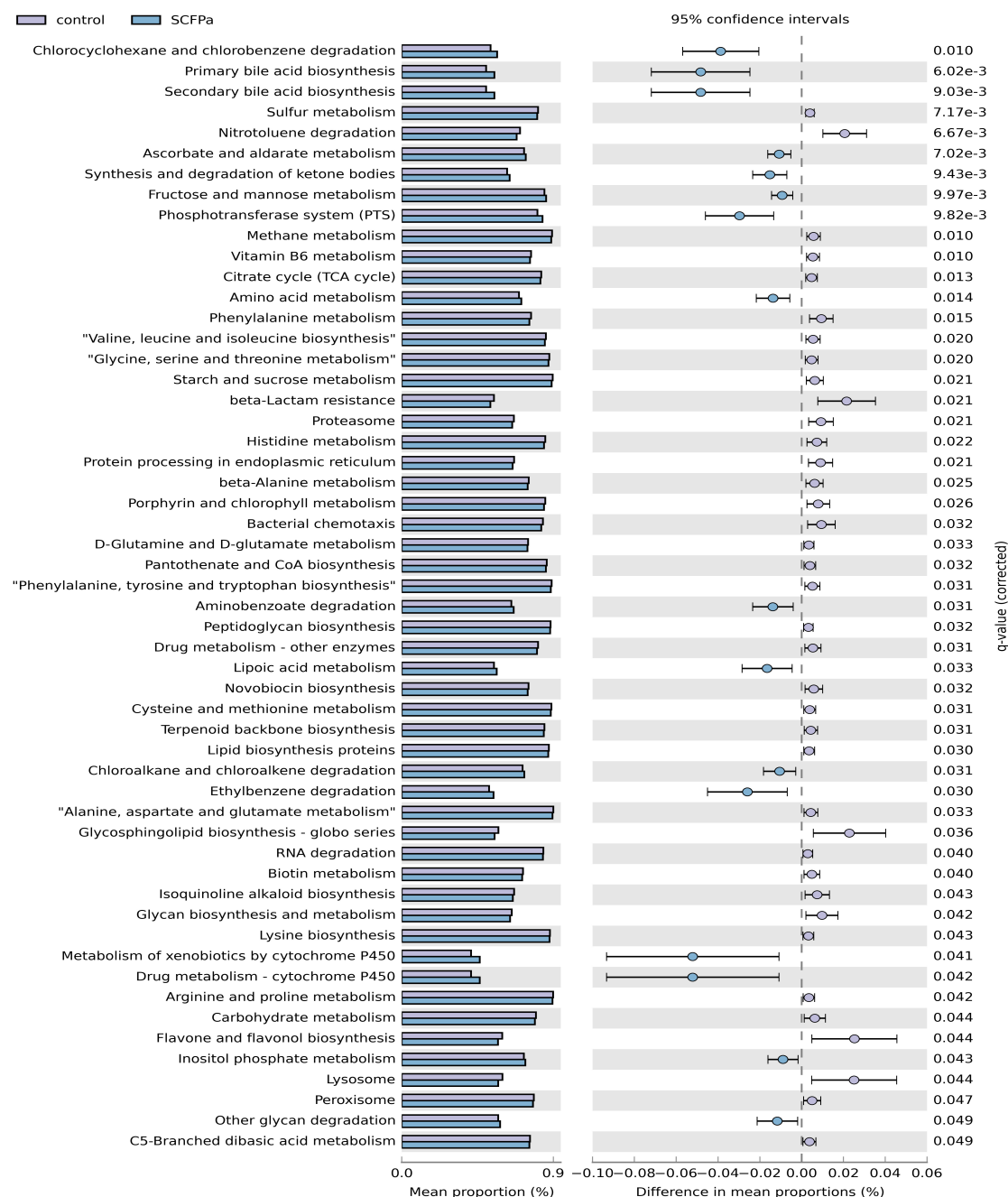


Figure 5-8. Differences in predicted functions of rumen solid microbiota between control and SCFPa groups during SARA. Functionalities of rumen solid microbiota were predicted using CowPi. Output was analyzed using STAMP following log_transformation and False Discovery Rate correction. Significant differences were considered as $P < 0.05$.

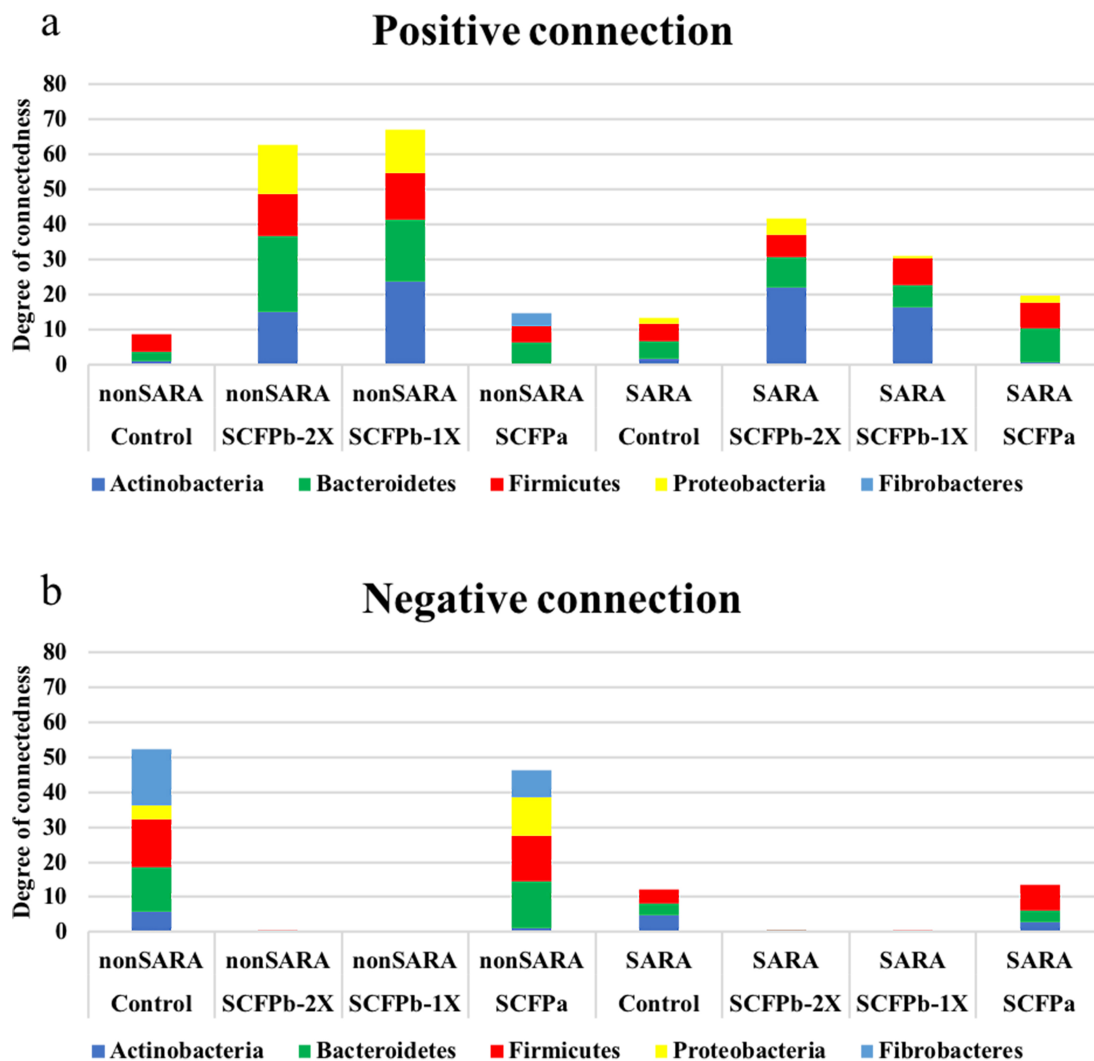


Figure 5-9. Microbial interaction networks in rumen solid digesta. The degree of connections for each phylum was normalized by dividing the total number of positive and negative edges observed for each phylum by their relative abundance in the community. a) Normalized positive connections for the dominant bacteria phyla within each treatment group during non-SARA (Pre-SARA1, Post-SARA1 and Post-SARA2) and SARA (SARA1/1, SARA1/2, SARA2/1, SARA2/2) stages. b) Normalized negative connections for the dominant bacteria phyla with each treatment group during non-SARA and SARA stages.

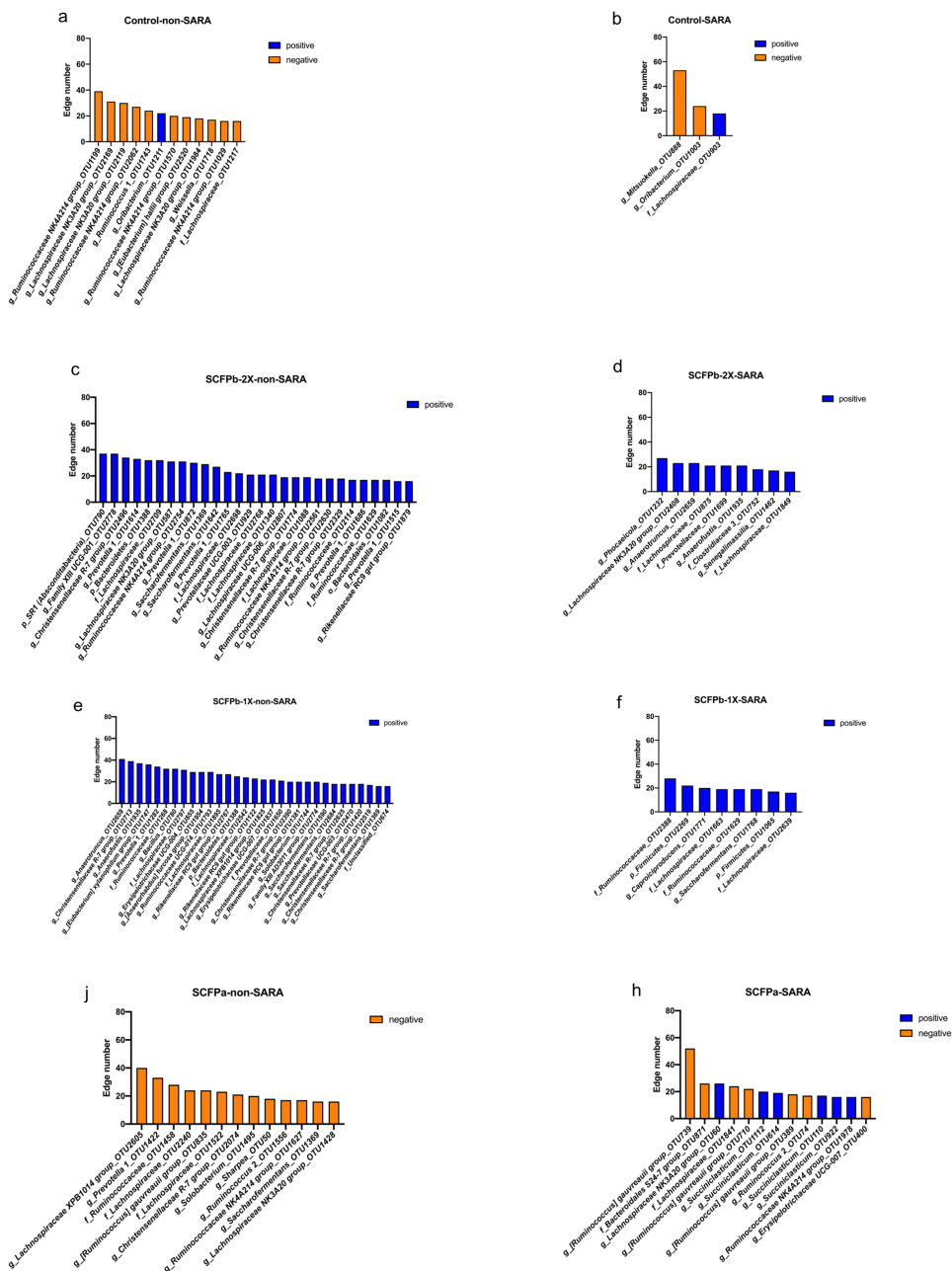


Figure 5-10. Distribution of hub taxa within each treatment group during non-SARA and SARA stages. Hub taxa were identified as OTUs with more than 15 connections with other members of rumen solid microbiota within each treatment group during non-SARA (Pre-SARA1, Post-SARA1 and Post-SARA2) and SARA (SARA1/1, SARA1/2, SARA2/1, SARA2/2) stages. a, b) control; c, d) SCFPb-2X; e, f) SCFPb-1X; j, h) SCFPa. Yellow color represents negative connections while blue color represents positive connections.

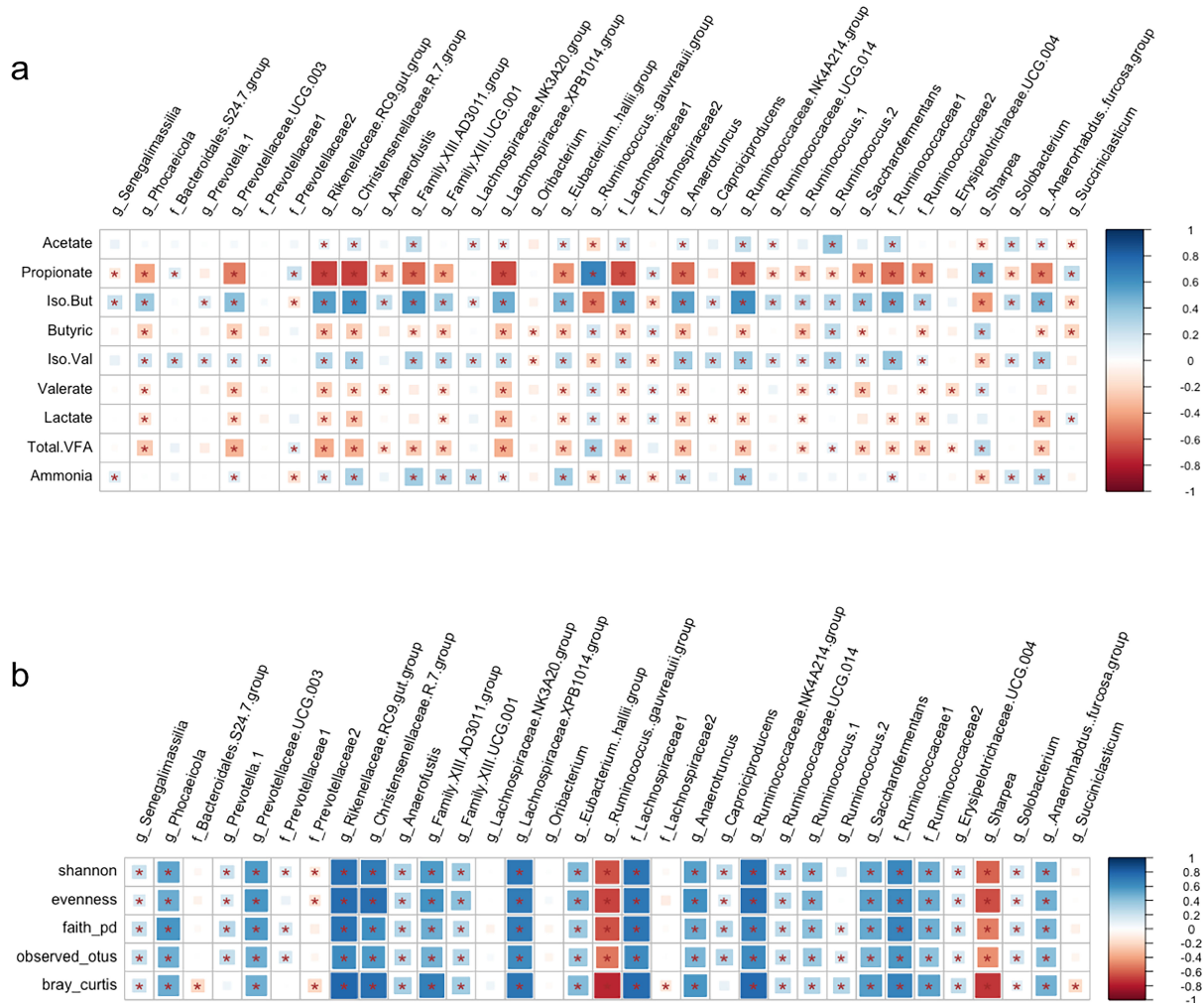


Figure 5-11. Correlation among hub taxa and rumen fermentation characteristics and biodiversity metrics of rumen solid microbiota. Spearman's correlation coefficient was used to explore the relationships between the relative abundances of rumen solid hub taxa and community α -diversity (Shannon, Evenness, Faith_PD and Observed_Features), β -diversity (Bray-Curtis dissimilarities) and rumen fermentation characteristics (VFAs and ammonia concentrations). * Indicates $P < 0.05$. The color ramp and the size of the squares indicate the type and strength of the Spearman's correlation coefficient (rho): rho = 1 showing strong positive correlation and rho = - 1 showing strong negative correlation between the two parameters.

5.5 Discussion

Postbiotics is a relatively new term that includes several bioactive constituents that have reported effects on improving rumen fermentation by increasing the abundances of cellulolytic bacteria and the synthesis of microbial proteins, as well as on stabilizing rumen microbes during nutrient challenges (Zhu et al., 2017; Tun et al., 2020; Vailati-Riboni et al., 2021). Rumen bacteria are the most abundant kingdom in the microbial community in dairy cows, and rumen solid-associated bacteria comprises more than 70% bacteria mass in the rumen (Craig et al., 1987; Mullins et al., 2013). These bacteria play key roles in degradation and fermentation of the digesta (Bickhart and Weimer, 2018). The relationship between the changes in the diet and the composition of the microbial community in the rumen has been widely studied. Our study revealed the effects of two commercial postbiotics from *Saccharomyces cerevisiae* fermentation on diversity, composition, predicted functionality and network structure of the bacterial community in rumen solid digesta during repeated grain-based SARA challenges.

5.5.1 Effects of Grain-based SARA challenges on diversity, composition and predicted functionality of rumen solid microbiota

A companion study showed that SARA was induced successively, as the SARA challenges increased the duration of rumen pH below 5.6 from 8 to 186 min/d (Khalouei et al., 2020). Also, the SARA challenges increased the concentration of rumen free LPS from 5,012 to 63,596 EU/mL and of blood serum amyloid A (SAA) from 19.76 to 71.08 µg/mL, which means SARA caused an inflammatory response (Guo et al., 2022). Gram-negative bacteria contain LPS in their cell wall, and research from Dai et al. (2019, 2020) found that LPS dosing increases the relative abundances of several gram-negative

bacteria involved in starch digestion and fermentation, as well as pH reduction in ruminal fermentation, including *Anaeroplasma*, *Ruminobacter*, and *Succinivibrio*, and LPS may be used by some acidosis related bacteria for growth, such as *Streptococcus bovis*, *Lactobacillus ruminis* and *Selenomonas ruminantium* (Dai et al., 2019; Dai et al., 2020). Thus, the increase of LPS concentrations in this study may be related to the increase in the rumen of amylolytic bacteria. It has been reported that a grain-based SARA reduces the rumen pH and the relative abundances of pH sensitive bacteria, such as fibrolytic bacteria, but increases the relative abundance of pH tolerant bacteria and amylolytic bacteria (Cherdthong et al., 2010; Mao et al., 2013; Petri et al., 2013; Plaizier et al., 2017). These changes in fiber-degrading and starch-degrading bacteria during SARA may also influence the rumen pH because of the lower fiber and higher starch digestion and fermentation. In our study, the dietary starch content was increased from 17.6 to 27.9% DM while the dietary NDF was decreased from 55.4 to 48.1% by the SARA challenges. Hence, we expected the similar changes in the populations of rumen bacteria as in previous studies.

Earlier studies reported that SARA reduces the richness and evenness of the rumen liquid microbiota (Fernando et al., 2010; Mao et al., 2013; Guo et al., 2023). However, some studies found a greater richness of the microbiota in solid rumen digesta during SARA (Lettat et al., 2012; McCann et al., 2016). In contrast, we observed a reduction in richness and evenness of the microbiota in solid rumen digesta during grain-based SARA. This indicates that less resilience and robustness in this community may be accompanied with the onset of SARA. The PERMANOVA and PERMDISP analyses of Bray-Curtis distances of microbial communities in rumen solid digesta also demonstrated that SARA

challenges had major effects on the differences in the taxonomic composition and beta diversity of the microbiota in this digesta.

In total, 14 phyla and 445 genera were identified in the microbiota in solid rumen digesta. Similar as reported in rumen liquid (Guo et al., 2023), the predominant phyla in the rumen solid digesta are Firmicutes and Bacteroidetes, which comprise more than 90% of this community in our study. The relative abundance of Firmicutes in rumen solid digesta was higher than that of Bacteroidetes. It has been reported that microbiota in solid rumen digesta play a major role in ruminal fiber digestion, as cellulolytic bacteria are more abundant in rumen solid digesta than in rumen liquid digesta and the most active fibrolytic enzymes are secreted by microbiota in rumen solid digesta (Williams et al., 1989; Michalet-Doreau et al., 2001). Bacteria in rumen solid digesta are more numerous compared with in liquid fractions, as more bacteria are attached to solid digesta rather than in liquid rumen digesta (> 60% vs. < 20% of total bacteria) (Yang et al., 2001). Martin et al. (2001) found that supplementation with barley decreased the rumen degradation rate of hay, and the fibrolytic activities (mainly polysaccharidase) of the bacteria in solid rumen digesta. It was expected that increased grain feeding increases the population of Firmicutes and decreases that of Bacteroidetes (Khafipour et al., 2009c; Huo et al., 2014). However, McCann et al. (2016) reported that the relative abundance of Firmicutes in the rumen decreased and that of Bacteroidetes increased on day 6 of a SARA challenge (McCann et al., 2016). Fernando et al (2010) observed a gradually increase in the population of Bacteroidetes in the rumen when beef steers were adapted to a high-concentrate diet (Fernando et al., 2010). In contrast with previous studies, we did not observe significant changes in relative abundances of Firmicutes or Bacteroidetes in

rumen solid digesta during grain-based SARA. This discrepancy may be due to different sampling locations in the rumen, as we collected samples from rumen solid digesta and they collected samples from rumen liquid digesta. Also, differences in experimental design, the technologies used, lactation progress and the severity of SARA among studies may have also contributed to the differences among studies.

The Lachnospiraceae family that belongs to the phylum Firmicutes is more abundant in solid rumen digesta compared with liquid phase from cows fed pasture (De Menezes et al., 2011). Lachnospiraceae has been reported to be associated with butyric acid production (Louis et al., 2010; Meehan and Beiko, 2014). Studies have been conducted on the correlation between the abundance of this family and feed efficiency, but their results are inconsistent (Guan et al., 2008; Myer et al., 2015; Li and Guan, 2017). These discrepancies among studies may be due to the lack of butyrate production by many members of this family (De Menezes et al., 2011). In our companion paper, SARA challenges increased the concentration of butyrate in the rumen fluid (Khalouei et al., 2020). Also, we observed that the abundances of the members from Lachnospiraceae either positively or negatively correlated with the butyrate concentration in the rumen fluid digesta. Fernando et al. (2010) found that the ratio of Firmicutes/Bacteroidetes was larger in hay-fed animals than in grain-fed animals (Fernando et al., 2010). Jami et al. (2014) reported a positive correlation between the Firmicutes/Bacteroidetes ratio and milk fat yield (Jami et al., 2014). However, SARA challenges did not affect the Firmicutes/Bacteroidetes ratio in our study. More data is needed to assess the SARA effect on the feed efficiency in our study.

Fibrobacteres, which is an important phylum of the cellulolytic bacteria, was reduced obviously during SARA in this study as the dietary starch content increased. Consistently, Lourenco et al. (2020) also reported that supplementation with grain decreases the abundance of Fibrobacteres in the rumen liquid digesta of beef calves (Lourenco et al., 2020). Fernando et al. (2010) detected an higher number of Fibrobacteres in rumen solid and liquid digesta in hay-fed animals than in grain-fed animals (Fernando et al., 2010). The reduction of the relative abundance of this phylum during SARA stages was expected as the dietary starch content was higher and that of fiber was lower in SARA diet compared with those during non-SARA stages. Actinobacteria, a gram-positive phylum, has also been shown to increase in rumen solid digesta during SARA in our study. In this phylum, the relative abundance of the most abundant genus *Bifidobacterium* from the family Bifidobacteriaceae, a lactic acid producer, has been shown to increase during SARA challenges (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2017; Monteiro and Faciola, 2020). Previous studies have also reported that the phylum Proteobacteria is more abundant in the rumen when animals are fed a high-grain diet (Fernando et al., 2010; Petri et al., 2013; Auffret et al., 2017). The relative abundance of Proteobacteria increased during SARA2 in our study, which is consistent with previous studies. The Proteobacteria phylum contains many pathogenic bacteria such as *Escherichia coli* and the relative abundances of several of these pathogens can be enriched when feeding grain diet (Bäumler and Sperandio, 2016; Auffret et al., 2017). Thus, the increase in the abundance of Proteobacteria may indicate the dysbiosis of the rumen microbiota and more pathogens may increase the risk of inflammation and metabolic dysfunction in the host (Petri et al., 2013; Shin et al., 2015).

Due to the changes of microbiota in solid rumen digesta that were observed during SARA challenges, differences in bacterial functionalities and metabolic pathway expressions were expected. It has been reported that feeding with more highly fermentable carbohydrates promotes the production of propionate and butyrate and lactate, and reduces the ratio of acetate-to-propionate in the rumen (Gozho et al., 2007, Li et al., 2016, Khalouei et al., 2020). Liu et al. (2022) found that cattle fed with grain diet had higher relative abundances of the starch-fermenting bacteria *Succinivibrionaceae* and *Succinimonas*, and higher relative abundances of the lactate-utilizing bacteria such as *Megasphaera* and *Acetobacter* (Liu et al., 2022). In our study, we found the relative abundance of genera *Succiniclasticum* was positively correlated with the propionate concentration in the rumen solid digesta. As a major succinate producer, the family *Succinivibrionaceae* competes with methanogens for hydrogen (Pope et al., 2011). Succinate is the precursor for propionate, which is a substrate for gluconeogenesis (Yost et al., 1977; Li and Guan, 2017). Hence, a higher ratio of propionate-to-acetate may reduce methane emissions from the rumen (Russell, 1998). In our companion paper, a higher propionate-to-acetate ratio during grain-based SARA challenges was observed. Gagen et al. (2015) found acetogens in both families of *Lachnospiraceae* and *Ruminococcaceae*, which can provide hydrogen and may increase when methane production is declined (Gagen et al., 2015). It has been reported that increases in rumen acetate can increase milk fat production as acetate is the major source of energy and substrate for milk fat synthesis in dairy cows (Urrutia and Harvatine, 2017; Urrutia et al., 2019). We determined that the relative abundances of ASVs from the *Lachnospiraceae* *NK3A20* group, *Ruminococcaceae* *NK4A214* group, *Ruminococcus* and

Ruminococcaceae groups were positively correlated to the acetate concentration in rumen fluid and with the diversity and richness of the microbial community in the rumen solid digesta. Thus, the growth of those bacteria got inhibited during grain-based SARA. Some researchers observed a positive relationship between less diversity in the rumen microbial community and feed efficiency (McCann et al., 2014; Myer et al., 2015; Li and Guan, 2017). Furthermore, Li and Guan (2017) showed that nitrogen metabolism activities were inhibited in efficient cows (Li and Guan, 2017). Similarly, we found predicted nitrogen metabolism pathways such as “alanine, aspartate and glutamate metabolism”, “lysine biosynthesis”, “phenylalanine metabolism”, “glycine, serine and threonine metabolism” and “valine, leucine and isoleucine biosynthesis” were inhibited during SARA. Valine, leucine and isoleucine are important contributor of microbial protein synthesis, and microbial protein are precursors for the synthesis of milk protein in the mammary gland (Allison et al., 1966; Xue et al., 2020). Thus, grain-based SARA challenges in our study may increase the feed efficiency but reduce the milk protein synthesis. However, predicted functions of rumen microbiome can be biased as the prediction is based on amplicon sequencing, further metagenomics is needed to prove this conclusion.

5.5.2 Effects of SCFP supplementation on diversity, composition and predicted functionality of rumen solid microbiota

The SCFP are commonly used as rumen fermentation modifiers. It has been reported that SCFP influence rumen fermentation through promoting the growth and functionality of fibrolytic bacteria and lactate utilizing bacteria, as well as increasing microbial protein synthesis, and reducing lactate accumulation (Callaway and Martin, 1997; Zhu et al., 2017). Different from what we found in rumen liquid microbiota, the effect of SCFP on

attenuating the dynamics of fluctuations in the main phyla in rumen solid microbiota during SARA challenges were not observed. This may be due to the different relative abundances of each phylum between rumen liquid and solid fractions (Petri et al., 2013; Brede et al., 2020), which responded differently to SCFP supplementation.

It has been reported that SCFP increases milk yield and feed efficiency in lactating dairy cows (Callaway and Martin, 1997; Hristov et al., 2010; Poppy et al., 2012). Xiao et al. (2016, 2018) found that the SCFP (XPC) increased ruminal butyrate concentrations and *Butyrivibrio* (belongs to Lachnospiraceae) richness in rumen liquid bacteria but did not influence the richness and diversity of bacteria in solid rumen digesta in calves (Xiao et al., 2016; Xiao et al., 2018). In disagreement with that, we observed that SCFPa supplementation promoted the growth of *Butyrivibrio* in rumen solid digesta during SARA challenges in current study. The abundance of *Butyrivibrio* in solid rumen digesta was positively associated with the dietary CP content, and this genus is known to have proteolytic and polysaccharide-degrading activities (Kelly et al., 2010; Liu et al., 2016). However, no changes in butyrate concentrations in rumen digesta were found due to SCFP supplementation in the companion paper (Khalouei et al., 2020), which agrees with the findings from Tun et al. (2020). These discrepancies may be due to the different primers used in these studies, and the animal's age as Xiao et al. (2018) used calves, and the diet composition.

Butyrate and propionate provide energy for the rumen epithelial cells, thus, higher concentrations of these VFAs may promote the development of rumen papillae and the absorption area for VFA (Meehan and Beiko, 2014). However, excessive accumulation of these VFA in the rumen will reduce the rumen pH (Gozho et al., 2005). It has been

demonstrated that SCFP promote the length and weight of rumen papillae (Lesmeister et al., 2004; Brewer et al., 2014; Xiao et al., 2016), and stabilizes the rumen pH during high-starch diet feeding or under grain-based SARA conductions (Allen and Ying, 2012; Li et al., 2016; Dias et al., 2018). In agreeewith that, our companion paper (Khalouei et al., 2020; Guo et al., 2022) reported that SCFPb-2X supplementation increased the rumen pH and reduced ruminal propionate and free LPS concentrations during grain-based SARA challenges, indicating the benefit of SCFP on reducing the adverse effects of SARA.

Different members of rumen microbiota utilize different substrates from the diet. Thus, a more diverse microbial community can be more efficient in using the available resources (Henderson et al., 2015). Tun et al. (2020) found that SCFP supplementation attenuated the reduction in richness and diversity of the rumen microbiota during SARA conditions (Tun et al., 2020). Although we did not find SCFP improved the richness in rumen solid microbiota as we did in rumen liquid microbiota, we found that the reduction in the Chao1 index of microbiota in rumen solid digesta was not significant during the SARA2 stage compared with non-SARA stages when SCFPb-2X was supplemented. This shows that the SCFPb-2X treatment attenuated the loss of the diversity during SARA challenges and helped to maintain the robustness and resilience of the rumen solid microbiota during these challenges, thereby stabilizing the ruminal microbial community during SARA challenges. Although there was a treatment effect on the beta-diversity as determined by PERMANOVA, the significant treatment effect obtained by PERMDISP analysis indicated that this effect may be influenced by the differences in the taxonomic composition within each group. Therefore, there is no evidence to prove the dissimilarity among microbial communities during SARA challenges.

The effect of SCFP supplementation on the functionality of the rumen microbiota has also been studied. Nitrogen metabolism in the rumen includes protein degradation that provides nitrogen for bacteria, and microbial protein synthesis that provides the majority of CP in the small intestine (Storm and Ørskov, 1983, Clark et al., 1992). Protein degradation in the rumen requires the combination of proteolytic and nonproteolytic enzymes and amino acid uptake by rumen microbes (Bach et al., 2005). Positive effects of amylases on protein degradation are reported in earlier studies (Assoumani et al., 1992; Tománková and Kopečný, 1995). As reported by Bach et al. (2005), the population of cellulolytic bacteria decreases with the reduction of ruminal pH, but that of proteolytic bacteria cannot be affected (Bach et al., 2005). Under high-concentrate feeding, starch-degrading bacteria predominate while the number of cellulolytic bacteria decreases, and, as a consequence, fiber digestion and protein degradation decreases (Mould and Ørskov, 1983; Cherdthong et al., 2010). Carbohydrates are the main energy resources for rumen bacteria, and they can also be used as carbon skeletons for microbial protein synthesis. The nitrogen uptake by the ruminal microbes can be improved when the readily fermentable carbohydrates increases (Stern and Hoover, 1979; Casper and Schingoethe, 1989; Cameron et al., 1991). When the rate of carbohydrates fermentation exceeds protein degradation rate, microbial protein synthesis decreases (Nocek and Russell, 1988). The reduction of ruminal nitrogen metabolism when cows are fed a high grain diet may be related to the increase in digesta passage rate in the rumen that reduces the proteolytic activity of rumen microbiota (Sullivan and Martin, 1999; Bach et al., 2005). Several studies have been conducted to determine the effect of SCFP on ruminal nitrogen metabolism. It has been reported that SCFP increase the nitrogen utilization by rumen

bacteria and reduce ammonia and methane emissions by dairy cows (Hristov et al., 2010). Tun et al. (2020) found that SCFP attenuated the reduction of nitrogen metabolism during SARA challenge (Tun et al., 2020). Our results, partly agree with these previous studies, as we found that SCFP promoted predicted “amino acid metabolism” and carbohydrates metabolism including “ascorbate and aldarate metabolism”, “fructose and mannose metabolism” and “inositol phosphate metabolism” during SARA challenges. Besides, we also observed that SCFP promoted the colonization of several fibrolytic bacteria, including *Lachnospiraceae* UCG-009, *Treponema*, unclassified *Lachnospiraceae*, unclassified *Ruminococcaceae* during the SARA challenges. Also, fibrolytic bacteria have high preference for ammonia (Bryant, 1973). Hence, the SCFP supplementation may have increased the nitrogen metabolism in our study.

Microbe to microbe association networks offer high-level insights into the changes of global structure of microbial communities (Peschel et al., 2021). We found that only the SCFPb-2X treatment maintained the characteristics of the network inside the rumen solid microbial community under grain-based SARA challenges, indicating a more resilient and stable microbial community during this treatment. Besides, members from *Lachnospiraceae* NK3A20 group, *Phocaeicola*, *Anaerotruncus*, *Anaerofustis*, *Senegalimassilis* and unclassified *Lachnospiraceae* played important hub roles in the network derived from the SCFPb-2X treatment during SARA and had strong positive associations with richness and diversity of the microbiota in solid rumen digesta. It is conceivable that increasing the abundance of above taxa will result in an increased diversity and richness of the microbial community.

5.6 Conclusions

Repeated grain-based SARA challenges reduced the richness and diversity and changed the β -diversity of the bacteria community in solid rumen digesta. Supplementation with SCFPb-2X attenuated the reduction in diversity of this bacterial community during the second SARA challenge. Grain-based SARA challenges also reduced the relative abundance of cellulolytic bacteria, such as *Fibrobacteres* in this digesta, but this was not attenuated by supplementation with SCFP. Grain-based SARA challenges also reduced the interaction among rumen bacteria and the diversity of hub taxa in solid microbiota. Supplementation with SCFPb increased the positive connectedness among rumen microbial members in solid rumen digesta and the diversity of hub taxa, and SCFPb-2X maintained the network of microbiota in solid rumen digesta. Grain-based SARA challenges inhibited the growth of several fibrolytic bacteria, while SCFP supplementation promoted the growth of fibrolytic bacteria during SARA challenges. These challenges also reduced the predicted nitrogen metabolism in the microbiota of solid rumen digesta, while SCFP supplementation attenuated this reduction. Due to the limitations of the 16s rRNA sequencing to classify taxa at the species level and that of the databases for rumen microbiome when using CowPi to predict the microbial functionality, further metagenomic assembly based (MAGs) approaches are needed to investigate the associations among microbes and the gastrointestinal health of the host animal, especially in ruminants that are exposed to nutritional and physiological stressors and challenges.

CHAPTER SIX

GENERAL DISCUSSION

6.1 General discussion

During the lifetime of dairy cows, they go through metabolic stressors such as SARA. SARA can compromise the homeostasis of the gut microbiome and immune functions. Therefore, the robustness and the resilience of the animals determine the capacity of them to resist the changes when exposed to or following the exposure to the stressors. In our study, a large number of cannulated dairy cows were used in randomized complete block design to avoid the crossover effect. Repeated SARA challenges were conducted to determine if the repeated exposure to the stressors caused more severe effects on the animals than a single SARA challenge. Moreover, we examined microbial community dynamics and predicted functions in both rumen liquid and solid fractions in relation to the SARA challenges, rumen pH, rumen VFAs and measures of inflammation, which is not common in rumen microbiome researches. In addition, two different SCFPs (XPC and NutriTek) were supplemented to the cows, to assess the effects of these postbiotics on assisting animals when they are exposed to the stressors such as SARA.

In this study, SARA challenges were conducted by increasing the dietary starch content from 18.6% to 27.9% of DM and decreasing the dietary NDF content from 34.9% to 28.2% of DM. SARA is considered successively induced when the rumen pH resulting from the SARA challenges drops between 5.2 and 5.6 for more than 3 h/d (Gozho et al., 2005; Gozho et al., 2007). In our companion paper, Khalouei et al. (2020) reported that the duration of rumen pH below 5.6 was increased from 8 to 186 min/d across the SCFP

treatments during SARA challenge, indicating the success, albeit moderate, induction of SARA (Khalouei et al., 2020). The duration of rumen pH below 5.6 was lower in the Control group in the second SARA than in the first SARA challenge, but was not different between the two SARA challenges in SCFP treatment groups. Thus, the repeated SARA challenge did not cause a more severe rumen pH depression than the first SARA challenge.

The SARA challenges decreased the NDF digestibility (61.1% vs. 49.0%), while they increased the apparent digestibility coefficient of starch (89.6% vs. 92.9%) (Khalouei et al., 2020). The decrease of the rumen pH was accompanied by an accumulation of VFAs (propionate (20.9 vs. 44.3 mmol/L) and butyrate (11.2 vs. 14.5 mmol/L)) and lactate (5.22 vs. 7.44 mmol/L) in the rumen, compared to the control diet (Khalouei et al., 2020). Therefore, less stimuli for rumination and less alkaline saliva secreted to neutralize the VFAs by the animals with less NDF content in the diet, and the amounts of VFAs produced with high starch diet may exceed the absorption capability of rumen epithelium and cause acidic environment in the rumen (Kleen et al., 2003; Zebeli et al., 2008; Penner, 2014; Jiang et al., 2017). Changes in rumen conditions cause the microbial changes in the rumen (Zebeli et al., 2012). Feeding a high-grain diet favors the growth of Proteobacteria, *Megasphaera elsdenii*, *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* populations (Fernando et al., 2010). *M. elsdenii*, *Prevotella spp.* and *S. ruminantium* are lactate-utilizing bacteria that can rapidly convert lactate to propionate, butyrate and valerate (Stone, 2004; Fernando et al., 2010). Therefore, SARA challenges are usually accompanied with higher concentrations of

propionate and valerate, and lower proportions of acetate, resulting in a lower acetate to propionate ratio (Stone, 2004).

Grain-based SARA usually increases the free LPS concentration in the rumen that results from the lysis of gram-negative bacteria due to the acidic environment, and can reduce the barrier function of the rumen epithelium (Gozho et al., 2007; Khafipour et al., 2009b; Zhang et al., 2019). In manuscript 1, we found that SARA increased the free ruminal LPS from 5,215 to 66,240 EU/mL, which suggests the increase in rumen LPS was not as high as in other studies. Besides, the LPS concentration in feces increased from 8,221 to 81,628 EU/mL during SARA, indicating increase in the bypass of carbohydrates of the rumen and the small intestine and fermentation in the hindgut. Proteobacteria and Bacteroidetes are major contributors to rumen free LPS pool, which was proved as we observed the increases in relative abundances of Proteobacteria in both rumen liquid and solid digesta during SARA. However, LPS were not detectable in peripheral blood. This may be because the translocation rate of LPS from rumen and hindgut was below the LPS clearance by the liver (Alexander and Rietschel, 2001; Eckel and Ametaj, 2016). Furthermore, we found that SARA challenges tended to increase the LTA concentration in peripheral blood from 0.65 to 0.70 ng/mL. This detectable concentration of LTA in peripheral blood in both pre-SARA and SARA may indicate a weaker immune response to LTA compared to LPS (Eckel and Ametaj, 2016). Both LPS and LTA can combine with cell surface receptors to stimulate the immune response in animals, such as the release of the proinflammatory cytokines and the production of APPs (Ginsburg, 2002; Eckel and Ametaj, 2016). Thus, we determined the inflammatory responses of dairy cows to grain-based SARA by detecting the concentrations of

proinflammatory cytokines and APPs in peripheral blood. We found that SARA increased the blood plasma concentration of SAA from 19.76 to 71.08 µg/mL and tended to increase the LBP concentration (10.01 vs. 12.77 µg/mL). However, SARA did not affect the blood plasma concentration of Hp and blood serum concentration of IL-6. That may be due to the moderate rumen pH depression resulting from the SARA challenge. Moreover, we did not find elevation in rumen papillae, which is an indicator of tissue inflammation (Chami et al., 2018), that may suggest we did not induce a severe inflammation in rumen papillae when we met the minimum requirements for induction of SARA. However, the sampling time of papillae was only two weeks before and two weeks after SARA challenge and that may miss the proper monitoring of MPO.

Rumen microbiota is known to be connected with rumen digestion, fermentation and absorption, metabolism and immune functions (Bickhart and Weimer, 2018; Zeineldin et al., 2018). The commensal microbiota competes with pathogen microbiota and restrict the growth of these pathogenic microbiota. Previous studies found that gut microbiota is involved in the maturation of the immune system by stimulating the maturation of gut-associated lymphoid tissue (Kim et al., 2011). Thus, the disruption of the gut microbiota, such as the changes in the composition and functionality of the microbial community, may affect systemic immune responses (Clavel et al., 2017; Wang et al., 2021). Many studies have revealed the association between SARA and disturbed microbiota and metabolites in the rumen (Khafipour et al., 2009c; Sekirov et al., 2010; Mao et al., 2013). It has been suggested that the depression of rumen pH during SARA may cause the imbalance of the rumen microbiota, and the shifts in the profile of rumen microbiome (Tajima et al., 2000; Khafipour et al., 2009c; Fernando et al., 2010; Hook et al., 2011). In

manuscript 2 and 3, we determined the changes of composition of the rumen bacteria in both liquid and solid digesta fractions. Similar as in previous studies (Fernando et al., 2010; Hook et al., 2011; Mao et al., 2013), we found that SARA challenges reduced the diversity and richness of rumen bacteria in both liquid and solid digesta fractions, thereby reducing the robustness and the resilience of microbial communities in rumen liquid and solid digesta so that increased the risk of diseases in animals.

The rumen pH is crucial for fiber degradation. Low rumen pH during SARA may cause the reduction of populations of pH sensitive bacteria, including cellulolytic bacteria, and the increases in the population of pH tolerant bacterium, such as amylolytic bacteria in the rumen (Mao et al., 2013; Petri et al., 2013). We identified the major phyla in the rumen bacterial community. Firmicutes and Bacteroidetes were most abundant phyla in both rumen liquid and solid digesta, and Firmicutes were more abundant in solid digesta compared with liquid digesta. It has been reported that microbiota in solid rumen digesta plays a major role in ruminal fiber digestion, as cellulolytic bacteria are more abundant in rumen solid digesta than in rumen liquid digesta and the most active fibrolytic enzymes are secreted by microbiota in rumen solid digesta (Williams et al., 1989; Michalet-Doreau et al., 2001). Bacteria in rumen solid digesta are more numerous compared with those in liquid fractions, as more bacteria are attached to solid digesta particles rather than present in liquid rumen digesta (> 60% vs. < 20% of total bacteria) (Yang et al., 2001). In contrast with other studies on the effect of SARA on foundation members (Fernando et al., 2010; McCann et al., 2016; Tun et al., 2020), the grain-based SARA in our study did not affect the relative abundance of Bacteroidetes in rumen liquid and solid digesta but tended to reduce that of Firmicutes in rumen liquid digesta. That

may be due to the relative moderate nature of the SARA induced in our study. Similar as in previous studies (Fernando et al., 2010; Lourenco et al., 2020), the relative abundance of Fibrobacteres, which is an important phylum of fibrolytic bacteria, was reduced in both rumen liquid and solid digesta during the SARA challenges. Besides, we detected that grain-based SARA reduced the abundances of the fibrolytic genera, such as members from Ruminococcaceae and Lachnospiraceae in both rumen liquid and solid digesta, also suggesting the reduced fiber degradation during SARA. That can be the reason of lower NDF digestibility observed during SARA in our companion paper (Khaloui et al., 2020). The SARA challenges increased the relative abundance of keystone members such as Proteobacteria in both of rumen liquid and solid microbial communities. This phylum includes many pathogenic bacteria, such as *Escherichia coli*, and the relative abundances of several of these pathogens can be enriched when a high grain diet is fed (Bäumler and Sperandio, 2016; Auffret et al., 2017). Thus, the increase in the abundance of Proteobacteria may increase the risk of inflammation and metabolic dysfunction in dairy cows (Petri et al., 2013; Shin et al., 2015), which is proved by the increase in inflammation in our study.

We then determined the predicted functionalities of the rumen bacteria and found that the grain-based SARA challenges increased the predicted “lipopolysaccharides biosynthesis” and “lipopolysaccharides biosynthesis proteins” metabolic pathways in rumen microbiota in liquid and solid digesta, agreeing with the observed increased LPS concentration in rumen liquid digesta. Furthermore, SARA inhibited the predicted nitrogen metabolism, including “penylalanine, tryrosine and tryptophan biosynthesis”, “alanine, aspartate and glutamate metabolism”, “lysine biosynthesis”, “phenylalanine

metabolism”, “glycine, serine and threonine metabolism” and “arginine and proline metabolism” in microbiota in both rumen liquid and solid digesta. This may indicate that SARA reduced microbial protein synthesis.

We examined the effect of supplementation with two SCFP (XPC and NutriTek indicate from Diamond V) on attenuating the adverse effects of SARA on ruminal and fecal LPS, plasma LTA, immune responses and rumen microbial changes in dairy cows. Similar as in Control group, the duration of rumen pH below 5.6 exceeded the threshold of 180 min/d for SARA in SCFPa and SCFPb-1X groups (Khalouei et al., 2020). However, this duration was only 79.3 min/d in SCFPb-2X group. Besides, only SCFPb-2X supplementation improved the NDF digestibility and lower total VFA concentration in the rumen compared with the Control group (Khalouei et al., 2020). These findings suggested that supplementation with SCFPb in 2X dose could stabilize the rumen pH and rumen fermentation, and this may promote the growth of fibrolytic bacteria in the rumen (Loor et al., 2016; Tun et al., 2020). The reason for better stabilizing rumen pH effect of SCFPb than SCFPa is that it contains more fermentable metabolites and propriety antioxidants including polyphenols (Diamond V). Although there was not a higher relative abundance of Fibrobacteres phyla found in the SCFP treatment groups compared with the Control group in rumen liquid and solid microbiota, we found SCFPb-2X promoted the growth of several fibrolytic bacteria, such as members from Ruminococcaceae and Lachnospiraceae in microbiota in rumen liquid and solid digesta. The relative abundances of these taxa are positively correlated with diversity of the microbial community, so that we also observed SCFPb-2X attenuated the reduction in the Chao1 and evenness of microbiota in rumen liquid digesta during SARA compared with

the Control treatment. A more diverse community has stronger stability and capability to fight against exogenous perturbations, such as SARA challenges (Lozupone et al., 2012; Konopka et al., 2015; Li and Guan, 2017). This shows that the SCFPb-2X treatment stabilizes the rumen microbial environment during SARA, so we found that the tended to reduce ruminal free LPS was lower in SCFPb-2X supplemented cows compared with the Control cows. However, SCFPb-2X did not influence the LPS concentration in feces. That may because SCFPb-2X transferred the starch fermentation to the hindgut and increased fecal LPS. Also, the SCFPb-1X and -2X treatments reduced the blood plasma LTA concentration compared with Control treatments (0.63, 0.65 vs. 0.73 ng/mL). This indicates that SCFPb, and especially the 2X dose, reduced this endotoxin from gram-positive bacteria in the rumen due to SARA challenges. Meanwhile, the SCFPb-2X treatment also reduced plasma SAA (23.14 vs. 39.81 $\mu\text{g/mL}$) and IL-1 β (3.71 vs. 11.94 pg/mL) compared to the Control treatment, suggesting the higher robustness of the cows with a higher diverse microbiota to combat the immunogenic compounds or pathogenic organisms that threaten host health during SARA (Coleman et al., 2020; Vailati-Riboni et al., 2021; Coleman et al., 2023).

Furthermore, the SCFPb-1X treatment reduced predicted “valine, leucine and isoleucine degradation”, and “phenylalanine metabolism” while SCFPb-2X reduced predicted “lysine degradation” metabolic pathways compared with the Control treatment in the microbiome rumen liquid digesta. It has been suggested that SCFPb supplementation aids in improving milk protein yield in dairy cows during nutritional challenges (Xue et al., 2020). However, no differences in predicted functionality were found between SCFPb supplementation and the Control treatment in the microbiome in

rumen solid digesta. That suggests that SCFPb supplementation may benefit bacteria in rumen liquid digesta more than bacteria in rumen solid digesta.

6.2 Limitations

Overall, compared with other studies, we used a large number of ruminal cannulated dairy cows in this study to meet the required statistic power, and conducted a randomly complete block design instead of the Latin square design to avoid carry over effect of treatments on cows. Also, we analyzed the changes in microbiota of both rumen liquid and solid digesta, so that we can explore which of these microbial community is more influenced by SARA and SCFP supplementation, and we obtained a more comprehensive overview of the rumen microbiota. However, there are some limitations in our study as well. There are still many other pro-inflammatory and anti-inflammatory cytokines such as TNF- α and IL-10, and the changes in APPs at mRNA level were not included in our study so that we may have missed the window for monitoring inflammatory markers. To better evaluate the effect of SARA and SCFP on rumen epithelium barrier functions, tight junction related proteins analysis and tissue biopsy are necessary which were also missed in this study. Besides, 16s rRNA sequencing used in this study relies on the sequencing for PCR amplicons of targeted marker genes so that the description of the microbiome is biased by the selected primers and inaccurate in identifying the microorganisms at the species and strain level. Furthermore, we only investigated the changes of bacteria but not other microbes that are also involved in rumen fermentation in the rumen. Therefore, the changes in rumen microbial functions under SARA challenges and SCFP supplements are also lack of enough evidences.

6.3 Future work

In order to better understand the effect of SARA and SCFP on rumen epithelium barrier functions and immune responses, more work needs to be done, such as, the impact on tight junction related proteins analysis including claudins, occludins and tight junction protein1 (Schneeberger and Lynch, 2004; Penner et al., 2011; Minuti et al., 2015); biopsy to evaluate the changes of rumen epithelial structure during SARA and SCFP supplementation; mRNA expression of APPs in the liver and immune function parameters, such as neutrophil and monocyte phagocytosis and oxidative burst (Coleman et al., 2023). Besides, multi-omics including metagenomic, metatranscriptomics and metabolomics approaches that capture the whole genomic and transcriptomic repertoire are needed to further investigate the associations among microbes and the digestion, fermentation, metabolism, and gastrointestinal health of the host animals, especially in ruminants that are exposed to nutritional and physiological stressors and challenges, such as SARA. These technologies can help us to find out which species or which genes are correlated to specialized metabolic pathways, and better inform the strategies to maintain gut health and improve animal productions.

CHAPTER SEVEN

CONCLUSIONS

Grain-based SARA challenges during early lactation increased free LPS concentrations in rumen fluid and feces, LTA in blood plasma and increased concentration of circulating SAA and tended to increase that of IL-1 β . Therefore, the moderate SARA challenges promoted an inflammatory response without causing ruminal epithelial tissue damage. SARA challenges also decreased the richness and diversity of microbial communities, inhibited the growth of some fibrolytic bacteria in rumen liquid and solid digesta. Besides, grain-based SARA challenges reduced the diversity of hub taxa, the significant associations among microbes and predicted nitrogen metabolism of the microbiome in rumen solid and liquid digesta. However, repeated SARA challenges were not more severe than single challenge.

NutriTek supplementation at the higher dose reduced the concentrations of free rumen LPS and blood LTA, SAA and IL-1 β compared to the Control-fed cows treatment during SARA challenges, indicating supplementation with NutriTek at the higher dose was more efficient to attenuate the negative effect of SARA challenges on immune responses in dairy cows than other supplements. NutriTek supplementation at the higher dose prevented rumen microbiota from losing their diversity during SARA challenges in rumen liquid but not in rumen solid fraction. All of SCFP supplementation reduced the fluctuation in rumen liquid microbial communities by stabilizing the proportions of foundation members and keystone members of the community during the periods that dairy cows were exposed to SARA challenges. Supplementation with NutriTek at the

higher dose, prevented fibrolytic and lactate utilizing bacteria from losing their OTU counts during SARA challenges, stimulated fiber degradation and reduced lactate accumulation in the rumen, thereby attenuating adverse effects of these challenges on rumen function. Meanwhile, NutriTek supplementation improved the predicted nitrogen metabolism that were erduced during SARA in the rumen microbiota. In genera, supplementation with NutriTek at the higher dose was more efficient to improve immune and digestive health in dairy cows, and it may have the largest effect on microbiota in rumen liquid digesta.

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