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The Effects Of Dietary Yeast Extracts On Rumen Microbiota And Fermentation In A Dual-Flow Continuous Culture Fermentation System

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THE EFFECTS OF DIETARY YEAST EXTRACTS ON RUMEN MICROBIOTA AND FERMENTATION IN A DUAL-FLOW CONTINUOUS CULTURE FERMENTATION SYSTEM

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Abstract

Dietary yeast supplements are a popular feed additive in ruminant diets as its inclusion can favorably alter the rumen microbiota and fermentation, and subsequently improve animal health and production. Yeast are a rich source of amino acids, peptides, organic acids, carbohydrates, lipids, vitamins, and minerals. Inclusion of nutrient-rich yeast and yeast extracts in the diet promote the growth of select groups of rumen microbiota, subsequently improving digestibility, volatile fatty acids, and pH profile. However, the large variability within yeast types and composition has created inconsistent results on these parameters, and further investigation into yeast product variability is crucial for understanding its use in ruminant diets.

The aim of this thesis was to evaluate six novel yeast extract treatments, different by origin and processing, and their influence on the abundance and diversity of rumen bacteria, protozoa numbers, digestibility, pH, and methane production. The study utilized a 6 x 6 Latin square design using dual-flow continuous culture fermenters (n = 6), including six 10-d periods consisting of 7-d of adaptation followed by 3-d of sample collection. Dietary yeast extract treatments were included at 4% on a dry matter (**DM**) basis of the total diet, where treatments and basal diets were combined and added to the fermenters twice daily in equal proportions (109 g DM total per fermenter/d). Treatments included 1) a Brewer's yeast extract with crude protein (**CP**) > 60% and a high degree of protein hydrolysis (**BrE**), 2) a blend of Brewer's yeast extract and Baker's peptone with CP > 65% and a mixture of high and low levels of protein hydrolysis and nucleotides (**BrEPN**), 3) a blend of Baker's yeast extract with CP > 50% and a mixture of high and medium levels of protein hydrolysis and nucleotides (**BENH**), 4) a blend of Baker's yeast peptone and yeast extract with CP > 65% and a mixture of high and low levels of protein hydrolysis and nucleotides (**BEPN**), 5) a blend of Baker's yeast peptone, Brewer's yeast autolysate, and Baker's yeast extract with CP > 50% and medium protein hydrolysis (**BEPBrA**), and 6) a blend of Baker's yeast extracts with CP > 60% and a mixture of medium and low levels of protein hydrolysis and nucleotides (**BENL**). Fermenter pH was recorded every minute using indwelling pH sensors, methane concentration was determined in triplicate twice daily at the time of feeding via a real-time gas analyzer system, and protozoa and bacteria samples were enumerated via microscopic and flow cytometry analysis, respectively. Bacterial DNA was extracted from harvested bacterial pellets for high-throughput sequencing of the 16S rRNA gene to determine bacterial abundance and diversity. Effluent samples were dried to determine DM disappearance and apparent digestibilities. Fermenter pH, methane, apparent digestibilities, and protozoa and bacteria enumerations were statistically analyzed via the PROC MIXED procedure of SAS. Fermenter pH, protozoa and bacteria counts, methane concentration, apparent digestibility, and bacterial abundance and diversity were not different across treatments. Yeast extract treatments provided at an inclusion rate of 4% on a DM basis, resulted in no differences on rumen microbiota and fermentation across treatments. Further examination of these yeast extract treatments in a dose-response study to determine efficacy as well as an *in vivo* study to determine their impact on animal health and production parameters could provide greater insight into the differences of yeast extract origin and processing in the rumen.

Dedication Page

I dedicate this thesis to my grandma, my guardian angel, and best friend whose belief in me never wavered. I love you and miss you every day.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADF	Acid detergent fiber
ADY	Active dry yeast
ALY	Autolysate
A:P	Acetate-to-propionate ratio
AUC	Area under the curve
BENH	Blend of Baker's yeast extract with CP > 50% and high and medium levels of protein hydrolysis and nucleotides
BENL	Blend of Baker's yeast extracts with CP > 60% and medium and low levels of protein hydrolysis and nucleotides
BEPBrA	Blend of Baker's yeast peptone, Brewer's yeast autolysate, and Baker's yeast extract with CP > 50% and medium protein hydrolysis
BEPN	Blend of Baker's yeast peptone and yeast extract with CP > 65% and high and low levels of protein hydrolysis and nucleotides
BrE	Brewer's yeast extract with CP > 60% and a high degree of protein hydrolysis
BrEPN	Blend of Brewer's yeast extract and Baker's peptone with CP > 65% and high and low levels of protein hydrolysis and nucleotides
CFU	Colony forming units
CP	Crude protein
DH	Degree of hydrolysis
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
HLY	Hydrolysate
LPS	Lipopolysaccharide
MOS	Manno-oligosaccharide
NCBI	National center for biotechnology information
NDF	Neutral detergent fiber
NMDS	Non-metric multidimensional scaling
NR	Not reported
OM	Organic matter
OTU	Operational taxonomic unit
PPC	Phosphorylated yeast protein concentrate
rRNA	Ribosomal ribonucleic acid
SARA	Sub-acute ruminal acidosis
SC	*Saccharomyces cerevisiae*
SEM	Standard error mean
TMR	Total mixed ration
VFA	Volatile fatty acid
WYC	Whole yeast cell
YCW	Yeast cell wall
YE	Yeast extract
YPC	Yeast protein concentrate

Chapter 1: The Effects of Dietary Yeast and Yeast-Derived Extracts on Rumen Microbiota and their Function

1.1 Abstract

This review outlines our current knowledge of dietary yeast supplements and their components and describes their effects on the rumen microbiota and function. Yeast supplementation in ruminant diets has been evaluated for its impact on fiber digestibility, rumen fermentation patterns, and nutrient use efficiency. The primary effect of yeast appears to be the stimulation in the growth of specific rumen bacteria populations, specifically, cellulolytic and lactate-utilizing bacteria. The relationship of lactate-utilizing rumen bacteria with dietary yeast, has been intensely researched due to their role in affecting rumen pH parameters and ultimately animal health. Yeast supplementation has been shown to modulate rumen pH, particularly when used in combination with high concentrate diets. This is likely due to yeast stimulating the growth of both lactate-utilizing bacteria and protozoa. Protozoa are shown to engulf starch, ultimately limiting starch fermentation capacity by lactate-producing bacteria. Despite these recognized benefits of yeast supplementation in the rumen, results are variable and inconsistent across published research, likely due to the lack of consistency among yeast strain, dose, and type. This review describes yeast and its cellular components and outlines the impact of yeast on 1) rumen bacterial diversity and protozoa numbers, volatile fatty acid profile, acetate: propionate ratio, lactate accumulation, pH, CH_4, NH_3-N, and feed digestibility, 2) dairy production parameters including dry matter intake and milk production, and 3) ruminant health.

1.2 Introduction

Currently, there are five different forms of feed additives used to influence the rumen microbiota in ruminant production systems: antibiotics, ionophores, probiotics, prebiotics, and phytogenics. Probiotics include dietary yeast supplements, which may increase production and nutrient use efficiency (Desnoyers et al., 2009) as well as improve animal health (Williams and Coleman, 1997). Yeast supplementation stimulates the proportion of cellulolytic bacteria in the rumen and enhances fiber digestibility (Chaucheyras-Durand et al., 2008). Additionally, yeast increases the proportion of lactate-utilizing bacteria, which supports healthier ruminal pH parameters (Chaucheyras-Durand et al., 2008). Moreover, these bacteria have been linked to changes in the acetate: propionate ratio (A: P) as they convert lactate to propionate via the acrylate pathway (Counotte et al., 1981). A decrease in the A: P is energetically beneficial since propionate serves as a H_2 sink, limits the amount of H_2 used for CH_4 production (Johnson and Ward, 1996). The aim of this literature review was to delineate the current knowledge of dietary yeast and yeast extracts and describe how they influence rumen microbiota and microbial function in dairy cows.

1.3 Nutritional Characterization of Dietary Yeast and Yeast Cellular Components

1.3.1 The Nutritional Profile of Yeast and Yeast Cellular Components

Yeast are single-celled eukaryotes classified in the fungi kingdom, generally ranging from 5-10 μm in size (Stone, 2006). Yeast cells are composed of two primary fractions, the cell wall and intracellular components. Whole yeast cells (WYC), after undergoing autolysis

or hydrolysis, create autolysates (ALY) and hydrolysates (HLY), respectively. ALY and HLY are further divided into yeast extracts (YE), the intracellular soluble components of yeast, and the insoluble yeast cell wall (YCW) fraction. Yeast-derived extracts, sometimes referred to as yeast cultures, include both the yeast biomass and metabolites produced during the fermentation process (Newbold and Rode, 2006). Yeast-derived extracts can include YE, ALY, and HLY (Shurson, 2018).

The YCW of *Saccharomyces cerevisiae* is a layered structure (Figure 1) comprised of polysaccharides and glycoproteins including manno-proteins, β(1,3) glucans, β(1,6) glucans, and chitin (Lipke and Orvalle, 1998; Table 1). Of the dry matter (DM) content of the *Saccharomyces cerevisiae* cell, about 15-30% can be attributed to the cell wall (Lipke and Ovalle, 1998). The outermost layer of the YCW is comprised of manno-proteins, highly glycosylated proteins that are approximately 50-95% sugar molecules (mostly mannose), and contain a backbone of α(1,6) linked mannose with attached α(1,2) and α(1,3) linked side chains (Lipke and Orvalle, 1998). The side chains are commonly referred to as manno-oligosaccharides (MOS). The inner components of the YCW are fibrous β-glucans which provide rigidity to the cell wall and consist primarily of β(1,3) glucans (50% of DM) and branched β(1,6) glucans (10% of DM), while chitin, the minor (1-3% of DM) constituent of YCW, serves to form a complex with the β-glucans adding to the insolubility of their fibers (Lipke and Orvalle, 1998). Chemically, the YCW of *Saccharomyces cerevisiae* is comprised of approximately 85-90% polysaccharides and 10-15% protein on a DM basis (Nguyen et al., 1998). The chemical composition of YCW, however, varies depending on the strain and species of the yeast (Bzducha-Wróbel et al., 2012).

The intracellular components of yeast (Table 2) are nutrient-dense and comprise peptides, amino acids, carbohydrates, lipids, vitamins, and minerals (Newbold and Rode, 2006). Yeast is rich in B vitamins, particularly vitamin B12 (Boulton and Quain, 2001). Isolated YE contain these nutrients as well; however, a key feature of YE is the highly concentrated peptide fraction, which can be greater than 50% of the total mass of the YE depending on processing method (Proust et al., 2019). The intracellular carbohydrates consist primarily of glycogen (16-20% on a DM basis) and trehelose (6-10% on a DM basis) as shown in Baker's yeast (Sols et al., 1971). These carbohydrate reserves are lower in Brewer's yeast due to fermentation conditions (Halasz and Lasztity, 1991) and have been reported to include only 9-15.6% (DM basis) glycogen (Boulton and Quain, 2001).

1.3.2 Forms of Dietary Yeast Supplements on the Market

To date, many commercialized yeast supplements contain a varying degree of live or dead cells of *Saccharomyces cerevisiae.* The commercial supplements containing live yeast are publicized as a dietary probiotic to stimulate the growth of the rumen microbiota. Most of these live yeasts are commercialized as an active dry yeast (ADY) form. The impact of ADY on rumen health has been extensively investigated; however, results have been inconsistent. Current ADY products contain greater than 1.5×10^{10} CFU/g of DM live yeast cells, maintaining their function of fermentation, and are marketed for their positive effects on fiber digestibility in the rumen (AlZahal et al., 2014). Primary modes of action of ADY include the utilization of dissolved oxygen, prolonging ADY's lifespan in the rumen, and creating a more favorable anaerobic environment for host microbiota (Chaucheyras Durand et al., 2008).

Yeast products containing dead *Saccharomyces cerevisiae* cells are commercialized as yeast cultures, containing the yeast as well as the fermentation medium in which they were grown (Newbold and Rode, 2006). Yeast cultures provide growth-promoting substrates for the rumen bacteria, such as B vitamins, organic acids, amino acids, and peptides (Newbold and Rode, 2006). During the process of fermentation where yeast utilize sugar, a variety of metabolites are produced which include peptides, alcohols, organic acids, and esters that may have favorable nutritional and health benefits for animals (Shurson, 2018). Moreover, the byproducts from the baking and brewing industry (Baker's and Brewer's yeast, respectively), are also nutrient-rich yeast supplement options (Stone, 2006). These yeast products can be purchased in liquid form but are commonly dried for ease of storage and feeding.

Recently, research focus has shifted toward the evaluation of ALY and HLY and their impact on the rumen microbiota. In assessing these products, the method and degree of hydrolysis are important factors to consider. The degree of hydrolysis reflects the number of broken peptide bonds, as well as the length of the peptides which can range from 2 to 20 amino acids that impart bioactive functionality (Mirzaeia and Mirdamadi, 2015). Processing methods impact the degree of hydrolysis, with one study reporting that autolysis of *Saccharomyces cerevisiae* yielded ALY with a higher degree of hydrolysis compared with enzymatic hydrolysis (Mirzaeia and Mirdamadi, 2015), ultimately yielding a greater number of peptides that can be more rapidly utilized by rumen bacteria. The bioactive peptides of ALY and HLY are also reportedly diverse, with ALY and HLY containing bioactive peptides with antimicrobial, antioxidative and immunomodulatory effects (Sánchez and Vázquez, 2017).

1.3.3 Relevance of Yeast and Yeast Cellular Components in Ruminant Diets

Dietary yeast supplementation in ruminants has not always yielded consistent production responses (Desnoyers et al., 2009), and many argue that this is primarily due to the variability of the yeast type used in the studies (Darabighane et al., 2019). However, a meta-analysis evaluating the impact of *Saccharomyces cerevisiae* supplementation on rumen fermentation parameters and milk production concluded that dietary yeast increases dry matter intake (DMI), total milk yield, ruminal pH, total VFA concentrations, and OM digestibility (Desnoyers et al., 2009). This conclusion was similar to the meta-analysis later published by Poppy et al. (2012), who examined the impact of *Saccharomyces cerevisiae* yeast culture supplementation on lactating dairy cattle performance and identified increases in DMI, total milk and milk component yield. The improved rumen fermentation and milk production was attributed to the stimulation of rumen cellulolytic and lactate-utilizing bacteria (Chaucheyras-Durand et al., 2008; Chaucheyras-Durand et al., 2012). It is important to note, however, that yeast inclusion and its influence on rumen protozoa numbers has been ambiguous. The inclusion of dietary yeast has resulted in increases in protozoal abundance (Kowalik et al., 2012; Shen et al., 2018; Chaucheyras-Durand et al., 2019), decreases in the abundance of certain protozoal genera (Silberberg et al., 2013; Jiang et al., 2017), or no differences in total protozoa numbers (Chung et al., 2011; Bayat et al., 2015). Nevertheless, the shift in the rumen microbiota community structure has ultimately been recognized to increase fiber digestibility (Guedes et al., 2008), total VFA production (Pinloche et al., 2013), and microbial protein synthesis (Moya et al., 2018), decrease lactate

accumulation (Silberberg et al., 2013) and methanogenesis (Lynch and Martin, 2002), and improve ruminal pH parameters (Bach et al., 2007).

1.4 Impact of Dietary Yeast and Yeast-Derived Extracts on Rumen Microbiota

1.4.1 Bacteria

Bacteria comprise 98% of total cells in the rumen and perform the largest portion of feed degradation in the rumen (Lin et al., 1997). The rumen bacteria composition is largely determined by the diet and its substrates. The majority of the bacterial taxa are categorized based on their substrate preference (i.e., cellulolytic, amylolytic, lactate-utilizing bacteria; Church, 1988). Cellulolytic bacteria, such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens,* and *Ruminococcus albus,* produce cellulases responsible for fermentation of cellulose, and these bacteria increase in abundance when diets are high in fiber (Mosoni et al., 2007). Cellulolytic bacteria are strict anaerobes that are sensitive to the presence of oxygen in the rumen and their abundance can be negatively impacted by a decline in ruminal pH, thereby negatively impacting fiber digestibility (Chaucheyras-Durand et al., 2012). Feeding high amounts of starch and sugars (% of DM) increases the abundance and activity of amylolytic bacteria, such as *Bacteroides amylophilus, Succinomonas amylolytica,* and *Streptococcus bovis*, yielding increased total VFA or lactate and lowering ruminal pH (Chaucheyras-Durand et al., 2008). *Streptococcus bovis,* which rapidly ferment carbohydrates into lactate, are positively correlated with an increased abundance of lactate-utilizing bacteria, such as *Selenomonas ruminantium,* and *Megasphaera elsdenii* (Church, 1988). Lactate-utilizing bacteria can alleviate the negative

effects of lactate on rumen pH by metabolizing lactate to VFA (Counotte et al., 1981). For example, in a study by Counotte et al. (1981), *Megasphaera elsdenii* metabolized lactate to butyrate prior to converting it to propionate via the acrylate pathway. Moreover, *Megasphaera elsdenii* had no dependency on carbohydrates, such as glucose or maltose, to complete this lactate conversion, and was more efficient at converting lactate per cell than *Selenomonas ruminantium* (Counotte et al., 1981). *Selenomonas ruminantium* are reliant on fermentation of glucose, sucrose, and xylose prior to converting lactate to propionate via the succinate pathway and do not utilize butyrate as an intermediary step, which is comparatively different than *Megasphaera elsdenii* (Counotte et al., 1981). Increases in the relative abundance of these species resulting from supplementation of yeast should increase the proportion of lactate utilization in the rumen, alleviating rumen pH and providing a greater concentration of VFA, such as propionate.

Feeding live yeast reportedly enhances fiber digestibility by removing trace amounts of O_2 entering the rumen with ingested feed particles during water intake and mastication (Chaucheyras-Durand et al., 2012). De-oxygenation of the rumen 1) provides a more favorable environment for anerobic microbes (i.e., cellulolytic bacteria) that lack the enzymes necessary for removing reactive oxygen species (Chaucheyras-Durand et al., 2012), and 2) enhances the binding affinity of anerobic microbes to feed particles (Jouany and Morjavi, 2007). In a study by Girard and Dawson (1994), the supplementation of a yeast culture stimulated the growth of *Fibrobacter succinogenes* S85 while reducing the lag-time to grow *Ruminococcus albus* 7, and *Butyrivibrio fibrisolvens*. Additionally, AlZahal et al. (2014) observed a 2-fold increase in *Fibrobacter succinogenes* and an 8-fold increase in *Ruminococcus albus* when dairy cows were supplemented with ADY

Saccharomyces cerevisiae (Biomate; AB Vista, Marlborough, United Kingdom; 8×10^{10} CFU/d). When lactating dairy cows were supplemented with 5.0 g/d of live *Saccharomyces cerevisiae* yeast (Sc47; Lesaffre Feed Additives, Marquette- Lez-Lille, France; 1 x 10^{10} CFU/g of DM) there was a 2.7-fold increase in *Ruminococcus* spp. compared with feeding either 0.5 g/d of live yeast or a control diet (Pinloche et al., 2013). In a study by Mosoni et al. (2007), live *Saccharomyces cerevisiae* (0.2 g/d; 4 x 10^9 CFU/d; CNCM I-1077; Levucell SC20; Lallemand Animal Nutrition, Blagnac, France) supplemented to sheep receiving a 50: 50 (forage: concentrate) diet resulted in an increased abundance of *Ruminococcus albus* and *Ruminococcus flavefaciens* (2 and 4-fold, respectively) with no difference in *Fibrobacter succinogenes* when compared to control sheep. This was similar to observations reported by Silberberg et al. (2013) who utilized the same animal model, yeast product, and dosage as Mosoni et al. (2007). They observed increases in *Ruminococcus flavefaciens* but not *Fibrobacter succinogenes.* Additional studies have observed increases (Vyas et al., 2014; Jiang et al., 2017) or no differences (Bayat et al., 2015) in rumen cellulolytic bacteria following yeast supplementation. The different outcomes of these studies on cellulolytic bacteria abundance are likely the result of differences in animal model, diet, yeast product, and the dose of the yeast.

The ability of yeast to influence the rumen microbiota has not solely been attributed to cellulolytic bacteria species, but also select lactate-utilizing bacteria. The metabolites produced by yeast supports the growth and function of *Megasphaera elsdenii* and *Selenomonas ruminantium* (Rossi et al., 2004). Some studies have resulted in an increased abundance of either one or both of these bacteria after supplementing yeast (Rossi et al., 2004; Pinloche et al., 2013) while the majority of studies resulted in no difference in their

abundance relative to control groups (Moya et al., 2009; Silberberg et al., 2013; Vyas et al., 2014; Jiang et al., 2017; Moya et al., 2018). Lactating dairy cows supplemented with 5.0 g/d of live *Saccharomyces cerevisiae* yeast (Sc47; Lesaffre Feed Additives, Marquette-Lez-Lille, France; 1 x 10^{10} CFU/g of DM) compared to either 0.5 g/d, or no yeast control, were shown to have a 3.1-fold increase in abundance of *Megasphaera elsdenii* and a modest increase in *Selenomonas ruminantium* (from undetected to 0.79% of the relative abundance) compared to either 0.5 g/d, or no yeast control (Pinloche et al., 2013). In an *in vitro* study investigating the impact of peptide fractions derived from *Saccharomyces cerevisiae* on the growth and metabolism of *Megasphaera elsdenii,* peptide fractions rich in lysine and histidine were the most effective at increasing the growth (18.5% increase in population size) and lactate utilization (74.1% increase in lactate disappearance) by this strain (Rossi et al., 2004). These results suggest that the amount at which yeast is supplemented impacts lactate-utilizing bacteria but also that growth promoting components of yeast can influence their utilization of lactate. Another study reported a 12-fold decrease in abundance of *Megasphaera elsdenii* concurrent with a 2.3-fold increase in *Streptococcus bovis* when ADY *Saccharomyces cerevisiae* (Biomate; AB Vista, Marlborough, United Kingdom; 8×10^{10} CFU/d) was supplemented to lactating dairy cows for 10 weeks (AlZahal et al., 2014). Results from the latter two studies highlight inconsistencies in bacterial response reported from yeast supplementation experiments. In addition, the determination of yeast efficacy is difficult due to the variability of yeast supplements utilized in the different studies. Further evaluation of differences in yeast strain are necessary to establish the true impact of yeast on rumen bacteria.

1.4.2 Protozoa

Protozoa range from 20-200 μm in size, are present in the rumen at amounts of 10^5-10^6 cells/g rumen content (Fonty and Chaucheyras-Durand, 2006), contribute 40% of the microbial N supplied, produce 60% of the total fermentation products (Church, 1988), and depending on diet, typically constitute approximately 50% of the rumen microbial biomass (Williams and Coleman, 1997). Currently, yeast is believed to stimulate the growth of rumen protozoa that engulf starch granules from the animal's diet and prevent the synthesis of lactate from amylolytic bacteria, subsequently outcompeting the bacteria for substrate (Williams and Coleman, 1997). Moreover, protozoa ferment starch to VFA at a much slower rate compared to bacteria, and utilize starch substrates that can be metabolized to lactate by rumen bacteria (Chaucheyras-Durand et al., 2008). The utilization of substrates by protozoa can increase VFA, which exhibit lower dissociative and acetogenic potential compared to lactate (Chaucheyras-Durand et al., 2008). This effect of yeast on protozoa metabolism leads to an increase in ruminal pH (Williams and Coleman, 1997) that may in turn affect fiber digestion. A study conducted by Shen et al. (2018) observed increased protozoa abundance when beef heifers were supplemented with a *Saccharomyces cerevisiae* fermentation product (18 g/d; NaturSafe; Diamond V, Mills Inc., Cedar Rapids, IA). Similarly, Chaucheyras-Durand et al. (2019) supplemented a combination of live yeast *Saccharomyces cerevisiae* and select yeast metabolites (undisclosed product information; intended to supply nutrients, vitamins, and growth factors) in the diets of lambs and observed an increase in rumen ciliate and small Entodiniomorphid protozoa compared to the control lambs. Furthermore, Jersey heifers had increases in *Entodinium* abundance when supplemented with yeast metabolites (Diamond V Mills XP®, Cedar Rapids, IA; 60

g/d) but not when supplemented with a live *Saccharomyces cerevisiae* (CNCM I-1077; Levucell SC; Lallemand Animal Nutrition, Blagnac, France; 10 g/d) or no yeast control (Kowalik et al., 2012). Furthermore, a decreased abundance of *Diplodinium* and an increased abundance of *Ophryscolex* and *Dasytricha* was observed in heifers supplemented with live yeast compared with those fed a no yeast control or yeast metabolites (Kowalik et al., 2012). Contrary to the previous studies, Silberberg et al. (2013) supplemented live *Saccharomyces cerevisiae* (4 x 10^9 CFU/d; CNCM I-1077; Levucell SC20; Lallemand Animal Nutrition, Blagnac, France) to the diets of sheep and observed that protozoa abundance of Entodiniomorphs (>100 μM) decreased following yeast supplementation. Furthermore, Jiang et al. (2017) reported decreased protozoa abundance in lactating dairy cows supplemented with a high dose of dead *Saccharomyces cerevisiae* (proprietary strain isolated from corn silage; Dupont Pioneer, Johnston, IA; 6.0×10^8 CFU/d) compared with a low dose of live *Saccharomyces cerevisiae* (proprietary strain isolated from corn silage; Dupont Pioneer, Johnston, IA; 5.7×10^7 CFU/d), a high dose of live *Saccharomyces cerevisiae* (proprietary strain isolated from corn silage; Dupont Pioneer, Johnston, IA; 6.0×10^8 CFU/d), and no yeast control. Additional studies observed no impact of yeast supplementation on rumen protozoa abundance (Chung et al., 2011; Bayat et al., 2015). These studies suggest that dietary yeast supplementation could contribute to the stimulation of protozoa numbers in the rumen, which has been postulated to exhibit a positive role on fiber digestibility through modulation of ruminal pH via lactate accumulation and a lowered rate of VFA synthesis (Chaucheyras-Durand et al., 2008). However, it is important to note that the ability of live yeast and yeast-derived extracts to stimulate total or select genera of protozoa varies greatly. Differences in the yeast product used, its composition,

and the dose supplied confounds inter-study result comparisons and complicates the assessment of yeast supplementation impacts on rumen protozoa activity and abundance. Further studies using the same application of yeast are necessary to determine yeast efficacy on the rumen protozoa populations.

1.5 Impact of Dietary Yeast and Yeast-Derived Supplements on Dairy Cow Performance

1.5.1 Rumen Environment and Function

Fiber digestibility in the rumen is impacted by four major factors: 1) plant structure and composition, which influence substrate availability for rumen bacteria, 2) population density of predominating fiber-degrading bacteria, 3) microbial factors that influence particle adhesion and hydrolytic enzyme complexes within the fibrolytic microbial communities, and 4) animal factors that increase nutrient availability, such as digesta kinetics, mastication, and salivation (Cheng et al., 1991). Studies that evaluated the efficacy of yeast supplementation on feed digestibility in the rumen have been contradictory; some report no change (Table 3; Moallem et al., 2009; Chung et al., 2011; Vyas et al., 2014; Bayat et al., 2015; Diaz et al., 2018; Moya et al., 2018) while others report an increase in digestibility (Guedes et al., 2008; Ferraretto et al., 2012). In a meta-analysis conducted by Desnoyers et al. (2009), yeast supplementation increased overall OM digestibility. Furthermore, the positive influence of yeast on OM digestibility decreased with the proportion of concentrate provided in the diet and increased with the proportion of NDF (Desnoyers et al., 2009). A study by Guedes et al. (2008) evaluated the effects of live *Saccharomyces cerevisiae* (Levucell SC 10 ME; 1 x 10^{10} CFU/g of DM) on fiber

digestibility in non-lactating cows fed corn silage with high and low levels of degradability and demonstrated a higher digestibility when yeast was fed at 1 g/d but not at 0.3 g/d in the low degradability group (Table 3). Similar results were observed in a study by Ferraretto et al. (2012), who supplemented live *Saccharomyces cerevisiae* (Procreatin-7; Lesaffre Feed Additives, Milwaukee, WI; 15×10^9 CFU/g) at two different dosages (2 g/d and 4 g/d) to lactating Holstein cows fed a high-starch diet (30% starch on a DM basis). Increased DM digestibility (4.3% vs 2.4%, respectively) and OM digestibility (3.9% vs 2.1%, respectively) were observed in response to feeding 2 g/d of yeast compared to 4 g/d (Table 3), however, the digestibility of NDF increased by 7.5% with the supplementation of 4 g/d of live yeast (Ferraretto et al., 2012). A study that analyzed the impact of providing a yeast culture (10 g/d; Yea-Sacc; Alltech Biotechnology Center, Nicholasville, KY) to lactating Holstein cows fed a high concentrate diet (42.75% on a DM basis) reported increased CP and ADF digestibilities when compared to a negative control (Table 3; Erasmus et al., 1992). These results highlight the variable impact of dosage and yeast type on feed digestibility. Although digestibility can be affected by yeast supplementation, diet composition and the proportion of fiber in the diet also contribute to the observed responses. An increase in feed digestibility is important for animal production and performance as it increases the passage rate and subsequently DMI in ruminants. Further examination of the confounding factors (i.e., yeast type, dose, and processing) that influence the efficacy of yeast is necessary to define its application in ruminants.

Yeast inclusion in ruminant diets has had variable effects on total VFA production and A: P. Several studies found positive effects of dietary yeast on ruminal VFA concentrations and A: P (Table 3; Guedes et al., 2008; Chung et al., 2011; Pinloche et al.,

2013; AlZahal et al., 2014) while other studies observed no differences (Table 3; Moallem et al., 2009; Moya et al., 2009; Thrune et al., 2009; Ferraretto et al., 2012; Silberberg et al., 2013; Vyas et al., 2014; Bayat et al., 2015; Diaz et al., 2018; Moya et al., 2018). A study evaluating the effects of live *Saccharomyces cerevisiae* (Levucell SC 10 ME; 1 x 10^{10} CFU/g of DM) resulted in significant increases of all major VFA with the largest increase being propionate, which resulted in a decreased A: P (Table 3; Guedes et al., 2008). The rise in total VFA concentrations was greatest when yeast was supplemented at 1 g/d compared to 0.3 g/d (Guedes et al., 2008). When evaluating the effect of ADY (Biomate; AB Vista, Marlborough, United Kingdom; 8 × 10^{10} CFU/d) in lactating dairy cows receiving high-grain diets, increases in total VFA and propionate, and a substantial reduction in A: P were observed (AlZahal et al., 2014). Similar results were also observed by Pinloche et al. (2013) when live yeast (0.5 g/d or 5 g/d) was provided to dairy cows. They observed increases in total VFA and propionate in cows supplemented with 5 g/d of live *Saccharomyces cerevisiae* (Sc47; Lesaffre Feed Additives, Marquette- Lez-Lille, France; 1 x 10^{10} CFU/g of DM) and a reduction in A: P for both levels of yeast inclusion, with 5 g/d resulting in the greatest reduction (Table 3; Pinloche et al., 2013). In the meta-analysis conducted by Desnoyers et al. (2009), yeast supplementation increased total VFA concentration without impacting the A: P. The energetically beneficial role of increasing VFA and decreasing A: P in ruminants promotes yeast as a valuable option for dietary supplementation in dairy cows. Further consideration of concentrate proportion in the diet and other factors, such as yeast product type and dose, need to be further evaluated.

Lactate accumulates in the rumen when an excess of highly fermentable grain (i.e., grains high in starch and sugars) is fermented by the rumen microbiota, mainly

Streptococcus bovis and *Lactobacillus* spp. (Chaucheyras-Durand et al., 2008). Lactate is the major driver of ruminal acidosis because of its high pKa (3.7) compared to the average pKa of the major VFA (pKa = 4.8-4.9; Chaucheyras-Durand et al., 2008). Live yeast supplementation of *Saccharomyces cerevisiae* in ruminants alters lactate accumulation in the rumen by 1) outcompeting lactate-producing microorganisms for sugars and 2) stimulating the metabolic function and growth by providing growth factors (e.g., B vitamins, amino acids, and organic acids) to lactate-utilizing bacteria (Chaucheyras-Durand et al., 1996). Decreased lactate concentrations in the rumen resulting from yeast supplementation have been observed (Chaucheyras-Durand et al., 1996; Guedes et al., 2008; Pinloche et al., 2013); however, others reported no effect (Table 3; Moya et al., 2009; Chung et al., 2011; Silberberg et al., 2013; Vyas et al., 2014). Pinloche et al. (2013) reported decreased ruminal lactate concentrations in dairy cows supplemented with 0.5 g/d and 5 g/d of live yeast compared to the control; however, lactate concentrations were the lowest when cows were fed the higher inclusion level (5 g/d) of yeast. The authors also observed a 3.1-fold increase in *Megasphaera elsdenii* and an increase in propionate concentrations with the addition of 5 g/d live yeast (Pinloche et al., 2013). The decrease in lactate and increase in propionate were likely the result of *Megasphaera elsdenii*, which utilize lactate as a substrate to produce propionate (Counotte et al., 1981). Guedes et al. (2008) supplemented dairy cows with live *Saccharomyces cerevisiae* (Levucell SC 10 ME; 1 x 10^{10} CFU/g; Lallemand Animal Nutrition, Montréal, Canada) at two doses, 0.3 g/d or 1 g/d, and observed lower rumen lactate concentrations compared to the control. Moreover, lactate concentrations were lower when cows were provided with 1 g/d of yeast compared

to 0.3 g/d or no yeast (Guedes et al., 2008). These results suggest that yeast dose influences lactate concentrations in the rumen and that higher dosages may provide greater benefits.

Yeast supplementation and its influence on ruminal pH has produced mixed results; but the majority of the research supports the hypothesis that reduced lactate accumulation and a greater microbial diversity lead to improvements in rumen pH (Chaucheyras-Durand et al., 2012). A rise in ruminal pH in response to yeast supplementation has been observed in numerous studies (see Table 3; Bach et al., 2007; Thrune et al., 2009; Guedes et al., 2008; Pinloche et al., 2013; Silberberg et al., 2013; Diaz et al., 2018), yet, several others observed no change (see Table 3; Mosoni et al., 2007; Moya et al., 2009; Ferraretto et al., 2012; Bayat et al., 2015). The meta-analysis by Desnoyers et al. (2009) concluded that yeast supplementation increases the overall rumen pH (0.03 units on average). Importantly, the higher pH response from yeast supplementation was mostly observed in conjunction with an increased DMI and a higher inclusion rate of concentrate in the diet (Desnoyers et al., 2009). The study by Guedes et al. (2008) evaluating live *Saccharomyces cerevisiae* (Levucell SC 10 ME; 1 x 10^{10} CFU/g; Lallemand Animal Nutrition, Montréal, Canada) supplementation in dairy cows at 0.3 g/d or 1.0 g/d, discovered that both doses of yeast increased mean ruminal pH compared to the control, but that rumen pH was not different between the two yeast treatments. However, Pinloche et al. (2013) observed a dose response when live *Saccharomyces cerevisiae* (BIOSAF SC 47; Lesaffre Feed Additives, Marquette-Lez-Lille, France; 1 x 10^{10} CFU/g) were supplemented to lactating Holstein cows at 0.5 g/d or 5 g/d. This study reported higher ruminal pH at both levels of yeast compared to the control, and 5 g/d yielded greater pH levels compared to 0.5 g/d (Table 3; Pinloche et al., 2013). These differences could be due to the difference in yeast type and

dose. Live *Saccharomyces cerevisiae* (1.5 g/kg of DM; NCYC 996, Procreatin-7, Phileo Lesaffre Animal Care, Campinas, Brazil) and MOS (1.5 g/kg of DM, β-glucans and mannan, Safmannan, Phileo Lesaffre Animal Care, Campinas, Brazil) both increased the ruminal pH in Holstein steers compared to the control steers, with no difference in response observed across yeast supplement types (Table 3; Diaz et al., 2018). A study by Vyas et al. (2014) utilized ADY and dead dried yeast (4 g/d; 1 x 10^{10} CFU/g; AB Vista, Marlborough, United Kingdom) supplemented in the diets fed to beef heifers. They observed an increased mean and minimum ruminal pH with supplementation of both yeast types compared to the control diet-fed heifers, but no differences were observed across the yeast treatment groups (Table 3). These results highlight potential benefits of dietary yeast on rumen pH and indicate that different yeast supplements may provide similar benefits. Additional studies further support the suggested positive impact of yeast supplementation on rumen pH based on the calculation of the length of time rumen pH is below 6.0, 5.8, and 5.6 (Table 3; Bach et al., 2007; Thrune et al., 2009; Silberberg et al., 2013; Moya et al., 2018). A study supplementing live *Saccharomyces cerevisiae* (5 g/d; 1 x 10^{10} CFU/d; CNCM I-1077; Levucell SC20, Lallemand Animal Nutrition, Montréal, Canada) to lactating dairy cows resulted in increased mean, minimum, and maximum ruminal pH, and decreased time spent below pH thresholds (5.6 and 6.0), calculated as area under the curve (AUC) in the rumen of cows fed yeast compared to control cows (Table 3; Bach et al., 2007). Similar results were demonstrated by Thrune et al. (2009), who supplemented live *Saccharomyces cerevisiae* (0.5 g/d; 1 x 10^{10} CFU/d; CNCM I-1077; Levucell SC20, Lallemand Animal Nutrition, Montréal, Canada) to lactating dairy cows and observed increases in mean, minimum, and maximum ruminal pH, and decreases in AUC (5.6, 5.8, and 6.0) with yeast

addition compared to the control cows (Table 3). When comparing the differences in rumen pH parameters between Bach et al. (2007) and Thrune et al. (2009), both utilized the same yeast product but at different doses, the difference between the control and yeast supplemented groups (nearly 2-fold) was more pronounced in results reported by Bach et al. (2007) than by Thrune et al. (2009). Additionally, the diet composition differed between the studies by Thrune et al. (2009) and Bach et al. (2007). Therefore, it is unclear, if the composition of the diet or the inclusion rate of yeast had the greater influence on ruminal pH. It was perhaps a combination of the two factors, given that the efficacy of yeast was shown to be greater in diets with higher concentrate proportions (Desnoyers et al., 2009). Furthermore, supplementation of live *Saccharomyces cerevisiae* (2×10^7 CFU/g of diet; CNCM I- 1077, Levucell SC; Lallemand SAS, Blagnac, France) in dual-flow continuous culture fermenters resulted in no changes in mean fermenter pH, however, the addition of yeast in combination with barley grain decreased the AUC (pH < 6.0), while the addition of yeast in combination with corn grain increased the AUC (at a pH threshold of 6.0) compared to the fermenters fed control diets (Table 3; Moya et al., 2018). Cumulatively, these studies suggest that the effect of yeast supplementation on ruminal pH may differ due to the composition of dietary concentrates. Additional considerations such as yeast product and dose should be considered when assessing factors that influence the rumen pH.

Another energetic fraction impacting rumen productivity is CH_4. Not only is CH_4 environmentally detrimental, but it also contributes to a 2-12% loss of energy in cattle relative to the energy content of the diet (Johnson and Ward, 1996). CH_4 is formed by methanogens, such as *Methanobrevibacter ruminantium* and *Methanosphaera stadtmanae,*

which have been identified as the primary species of methanogens in dairy cows fed a total mixed ration (Whitford et al., 2001). Methanogens remove H_2 and CO_2 build-up in the rumen during the process of carbohydrate fermentation and thereby contribute to the process of maintaining rumen homeostasis. Nutritional strategies have been explored for their potential to reduce CH_4 accumulation in the rumen, and some studies have evaluated how dietary yeast supplements impact CH_4 concentrations. The majority of studies, however, were conducted *in vitro* and there is very little information regarding the effect of yeast on H_2-transfer mechanisms and methanogenesis (Chaucheyras-Durand et al., 2008). It was originally hypothesized that yeast promotes a shift from methanogenesis to acetogenesis through stimulation of acetogenic bacteria, which are then capable of outcompeting methanogens in their H_2 utilization (Chaucheyras-Durand et al., 2008). This hypothesis was further explored by supplementing live and dead *Saccharomyces cerevisiae* (1×10^8 CFU/mL) which increased H_2 utilization by acetogenic bacteria (70% H_2 utilization) on culture plates when yeast were supplemented, and the control treatment resulted in H_2 being directed away from acetogenic bacteria (19% H_2 utilization) to methanogens (72% H_2 utilization; Chaucheyras-Durand et al., 1995). More recently, Ogunade et al. (2019) observed increases in relative abundance of methanogens when Holstein steers were supplemented with 15 g/d live yeast (Peloton live yeast product; PMI; Arden Hills, MN). These researchers attributed the increase in methanogens to the increase in relative abundance of cellulolytic bacteria, which supplies H_2 to methanogens for growth (Ogunade et al., 2019). However, despite the increase in abundance of methanogens, the increase in fiber digestibility and feed efficiency from the growth of cellulolytic bacteria will likely reduce the amount of CH_4 produced per unit of milk or meat, ultimately

improving CH_4 following the supplementation of yeast (Ogunade et al., 2019). Further research on CH_4 production *in vitro* was made by Lynch and Martin (2002), who found a reduction in CH_4 following a 48 h cultivation of rumen bacteria with alfalfa and live yeast (Table 3; 0.35 or 0.73 g/L of rumen inoculum using batch culture; Saf Agri PMX70SBK; Milwaukee, WI). A study by Oeztuerk et al. (2016) evaluated hydrolyzed WYC (HWYC; 0.25 or 0.75 g/d; Progut® Rumen; Suomen Rehu Oy, Helsinki, Finland), less-hydrolyzed WYC (LHWY; 0.25 or 0.75 g/d; Suomen Rehu, Espoo, Finland), and YCW (0.25 or 0.75 g/d; Bio-Mos®; Alltech Inc, Nicholasville, KY, USA) using the rumen simulation technique (i.e., Rusitec). They observed lower CH_4 concentrations (mmol/d) when HWYC was supplemented compared to the control but did not observe any differences with the addition of LHWY or YCW (Table 3). The few studies that investigated the effect of yeast on rumen CH_4 production were included in a recent meta-analysis by Darabighane et al. (2018), who reported no effect of yeast supplementation on CH_4 concentrations or CH_4 concentration as a proportion of DMI in both dairy and beef cattle. Darabighane et al. (2018) suggested that the current gap in knowledge of yeast and its role in CH_4 production should be further evaluated through testing different yeast doses, yeast strains, and yeast products, as well as the use of different experimental designs.

Another route of nutrient release in the rumen is through proteolysis. Degradation of feed particle proteins in the rumen produces peptides and amino acids, and the latter are taken up by the rumen microbiota for microbial protein synthesis or are further deaminated to keto acids, and metabolized to VFA, CO_2 and NH_3 depending on energy availability (Bach et al., 2005). Microbial protein comprises 50-80% of absorbed protein in the small intestine (Storm and Ørskov, 1983) and is an important nutritional substrate for the

ruminant. The ruminal NH_3-N concentration can be used to predict the efficiency of dietary N incorporation into microbial protein (Bach et al., 2005) and is therefore used when examining feed efficiency. Studies using dietary yeast supplements have reported reduced ruminal NH_3-N concentrations (see Table 3; Moallem et al., 2009; Pinloche et al., 2013; Oeztuerk et al., 2016; Moya et al., 2018). Pinloche et al. (2013) observed a decrease in ruminal NH_3-N concentrations when lactating dairy cows were supplemented with 0.5 g/d or 5.0 g/d of live yeast compared with control cows. Following the supplementation of live *Saccharomyces cerevisiae* (Biosaf, Lesaffre; 0.25 g/kg of DM) in dairy cows, a reduction in ruminal NH_3-N was observed compared to control cows (Table 3; Moallem et al., 2009). Similarly, Diaz et al. (2018) observed a reduction in rumen NH_3-N concentration after supplementing 1.5 g/kg of DM of live *Saccharomyces cerevisiae* (NCYC 996, Procreatin-7, Phileo Lesaffre Animal Care, Campinas, Brazil) or 1.5 g/kg of DM of MOS (β-glucans and mannan, Safmannan, Phileo Lesaffre Animal Care, Campinas, Brazil) in Holstein steers receiving live yeast or MOS compared to the negative control steers (Table 3). Similar results were observed in continuous culture systems supplemented with live *Saccharomyces cerevisiae* (CNCM I- 1077, Levucell SC; Lallemand SAS, Blagnac, France; 2 x 10^7 CFU/g of DM; Moya et al., 2018). Conversely, there have been multiple studies showing no effect of yeast supplementation on NH_3-N or microbial protein synthesis (Table 3; Guedes et al., 2008; Moya et al., 2009; Thrune et al., 2009; Chung et al., 2011; Vyas et al., 2014; Bayat et al., 2015). Hypotheses regarding fungal additives, such as yeast, and their efficacy on NH_3-N in the rumen have been postulated, and studies addressing this question suggest that NH_3-N responses are relatively small or non-significant, and may have little biological significance (Wallace and Newbold, 1995).

1.5.2 Dairy Cow Intake and Milk Production

Improvement of feed efficiency in dairy cattle is a primary target to advance sustainability goals of the dairy industry. Increased digestibility of feed can increase DMI of cows, which can allow for greater milk production overall. Management of factors that impact DMI are crucial for milk production and overall animal performance including yeast and yeast-based supplements having been evaluated for their effects on feed digestibility, DMI, and milk production parameters. A study by Moallem et al. (2009), where live yeast was supplemented in the diet of dairy cows during the hot summer months, when DMI is typically suppressed, reported a 2.5% increase in DMI as well as a 4.1% increase in average milk yield when yeast was fed compared to a control (Table 3). Erasmus et al. (1992) observed increased DMI when a yeast culture (10 g/d; Yea-Sacc; Alltech Biotechnology Center, Nicholasville, KY) was provided to lactating Holstein cows compared to a negative control (Table 3). Similar effects of live yeast on DMI and milk yield were also reported in the meta-analysis by Desnoyers et al. (2009), in which they identified a positive linear association of DMI and milk yield in response to yeast supplementation. A meta-analysis by Poppy et al. (2012) reported increases in total milk yield (1.18 kg/d) and DMI (0.62 kg/d) when early lactation dairy cows were provided YC. However, it is important to note that this analysis indicated a decrease in DMI (0.78 kg/d) in late lactation cows compared to early lactation cows (that showed increased DMI), suggesting that additional factors, such as stage of lactation, affecting DMI are still relevant regardless of yeast inclusion.

Additionally, milk components are an important indicator of dairy cow performance. The meta-analysis by Poppy et al. (2012) highlighted the responses of milk

components from supplementation with yeast cultures from *Saccharomyces cerevisiae* reporting increases in fat-corrected milk (3.5%), energy-corrected milk (kg/d), milk fat yield (kg/d), and milk protein yield (kg/d), but observed no differences in milk fat (%), and milk protein (%), which is similar to the meta-analysis by Desnoyers et al. (2009) who examined the impact of live yeast. Nocek et al. (2011) assessed the effects of a yeast culture (56 g/d; A-Max Yeast Culture Concentrate; Vi-COR, Mason City, IA) and a yeast culture and HLY mixture (28 g/d; Celmanax; Vi-COR, Mason City, IA) supplemented in the diets of dairy cows and observed increases in milk yield (kg/d), fat-corrected milk (3.5 %; kg/d), energy-corrected milk (kg/d), milk protein (%), milk fat yield (kg/d), and milk protein yield (kg/d) in both yeast treatments compared to a control (Table 3). In a study by Tristant and Moran (2015), lactating dairy cows were supplemented with a yeast culture (25 g/d; Yea-Sacc Farm Pak; Alltech Inc. Nicholasville, KY; 1.07 s 10^8 CFU/g) and observed increases in total milk yield (kg/d), energy corrected milk (kg/d), milk protein yield (kg/d) and lactose concentration, and decreases in milk fat yield (kg/d; Table 3). Conversely, no differences in milk yield or milk components were observed when lactating Holsteins cows were supplemented with 2 or 4 g/d of live yeast (Procreatin-7, Lesaffre Feed Additives, Milwaukee, WI; Ferraretto et al., 2012). These results on milk components and yield in dairy cows indicate that different forms of dietary yeast can elicit changes in certain milk components and overall milk yield. However, the vast number of additional variables that impact DMI, milk yield, and milk components across the different studies make the impact and response efficacy of dietary yeast on these parameters difficult to conclude, and therefore more studies are necessary to determine whether DMI and milk production are

affected similarly across a variety of yeast products given the variability in type, species, and dosage between studies.

1.5.3 Animal Health

Apart from the influence of yeast supplementation on rumen health, post-absorptive health benefits of yeast supplementation in ruminant diets have also been observed. Yeast supplements containing the YCW, such as ALY and HLY, contain the cell wall component of MOS. The presence of MOS was shown to exert prebiotic properties reducing inflammation and infection by providing growth-promoting factors (Spring et al., 2015). Moreover, because MOS have antioxidant and anti-mutagenic properties, they can prevent attachment of harmful bacteria in the gastrointestinal tract subsequently improving the intestinal mucosa and providing immune defense (Spring et al., 2015). β-glucans (i.e., YCW components) also exhibit immunomodulatory effects by enhancing the innate immune system in animals, which in part could be due to immune cells (i.e., macrophages) that possess receptors for β1,3 and β1,6-branched glucans (Shurson, 2018). Similarly to MOS, β-glucans can bind bacterial pathogens to prevent attachment and colonization in the gastrointestinal tract (Shurson, 2018). Ruminants receiving a high grain diet are subject to increased microbial endotoxin release of the cell wall component lipopolysaccharides (LPS) leading to an inflammatory response, which can circumvent select acid-resistance mechanisms, and ultimately increasing pathogenic virulence (Chaucheyras-Durand et al., 2012). Diaz et al. (2018) evaluated the effect of 1.5 g/kg of DM of live *Saccharomyces cerevisiae* yeast (NCYC 996, Procreatin-7, Phileo Lesaffre Animal Care, Campinas, Brazil) or 1.5 g/kg of DM of MOS (β-glucans and mannan, Safmannan, Phileo Lesaffre

Animal Care, Campinas, Brazil) supplements in dairy cattle diets on plasma concentrations of LPS and serum Amyloid A and determined that, although neither supplement affected the LPS concentration in the rumen or duodenal fluid, both affected the plasma concentrations of LPS and serum Amyloid A (Table 3). The authors attributed these results to reduced translocation of LPS and serum Amyloid A in the blood, reducing inflammation caused by high-grain diets (Diaz et al., 2018). Additionally, there is research to suggest that providing MOS in the diet of dairy cows can enhance the specific immunity of cows that have been vaccinated for viral diseases, and subsequently enhance the immunity of calves receiving colostrum. A study by Franklin et al. (2005) supplemented MOS (10/d; Alltech Inc., Nicholasville, KY) to dry cows that received vaccination against rotavirus, and determined that blood serum titers at calving were greater in cows supplemented with MOS compared to a negative control. Moreover, calves that received colostrum from MOS supplemented cows had a tendency to have greater blood serum titers and serum protein concentrations compared to calves from cows fed a negative control (Table 3; Franklin et al., 2005). These results suggest that supplementation with MOS can provide greater immunity to cows vaccinated against viral diseases, and that milk colostrum could provide greater passive immunity to calves. However, the effects of supplementing ALY and HLY containing both β-glucans and MOS on animal health have been difficult to determine as the quantity of β-glucans and MOS that bypass the rumen is not fully understood (Ballou et al., 2019). Nevertheless, evidence suggests that one or both of these components reach the gastrointestinal tract at a given point, as supplementation has shown to reduce stress-related disorders, improve mammary health, and increase overall milk production (Ballou et al., 2019). The potential for yeast components to influence animal health, specific

immunity, and alleviate acidosis and inflammation caused by high-grain diets needs further exploration to fully understand their efficacy in ruminants.

1.6 Conclusion

Yeast and yeast-derived supplements are considered beneficial in ruminant diets because they promote the growth of favorable microbes in the rumen, alleviate depressed ruminal pH and enhance fiber digestibility by removing trace amounts of oxygen in the rumen. There is a growing body of knowledge characterizing the effects of dietary yeast and yeast-derived extracts on specific rumen microbiota (i.e., cellulolytic and lactate-utilizing bacteria) and how they affect rumen function such as fiber digestibility, N utilization, ruminal pH, VFA and methane. However, the complex interactions of these supplements with rumen metabolic pathways are still largely unexplored, and further evaluation of how yeast strains and their components influence the rumen microbiota is crucial for understanding microbial shifts and subsequently their metabolites produced. The greatest challenge with yeast supplementation thus far has been the lack of consistent responses. Further studies using the same experimental design, methodology, yeast product and dose are necessary to establish a firm understanding of supplementation efficacy on rumen microbiota, fermentation, and ultimately production parameters. Careful consideration of both diet and environmental factors among studies can lead to a greater understanding of yeast supplementation as a viable dietary feed additive in ruminants.

1.6.1 Hypothesis

We hypothesized that the inclusion of dietary yeast extracts of different origin and processing effects would differentially impact the rumen microbiota and rumen fermentation parameters (Chapter 2). We further hypothesized that 1) yeast extract treatments derived from Brewer's yeast would result in lower CH_4 concentrations, and 2) the yeast extract treatment with the greatest number of yeast cell components would have the greatest impact on the abundance and diversity of cellulolytic and lactate-utilizing bacteria from the diversity of growth-promoting factors, and subsequently have improved fermenter pH and digestibility from these increases in cellulolytic and lactate-utilizing bacteria.

1.6.2 Objectives

Therefore, the objectives of the current study were to evaluate six different yeast extract treatments on rumen microbiota and fermentation that contain one or a combination of 1) yeast origin (Brewer's and Baker's), 2) processing method (low, medium, or high degree of protein hydrolysis), 3) yeast component (extract, peptone, and autolysate), and 4) the inclusion or absence of yeast nucleotides. The specific aims of our study were to 1) assess the changes in rumen bacterial relative abundance and diversity, especially that of cellulolytic and lactate-utilizing bacteria, and 2) identify changes in rumen fermentation as a result of these changes in rumen bacteria, such as fermenter pH, digestibility, and methane concentration.

1.7 Acknowledgements

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Table 1.1. Nutritional composition of the extracellular (i.e., cell wall) components of *Saccharomyces cerevisiae*.

Type	DM (%)											Reference
	Total DM (%)	B1,3	B1,6	Manno-protein	Chitin	Polysaccharide	Protein[2]	Carb	N	Glucose	Mannose	
Sp.1117	29	NR	NR	24	3.4	86.5	13.5	NR	NR	NR	NR	Nguyen et al., 1988
No. 1 (Brewer's yeast)	28	35	11	54	NR	NR	NR	NR	NR	NR	NR	Bzducha-Wróbel et al., 2012
Sp.102 (Baker's yeast)	26	31	6	63	NR	NR	NR	NR	NR	NR	NR	Bzducha-Wróbel et al., 2012
Sp. 1109	22	NR	NR	NR	NR	NR	NR	99	0.13	98.5	1.5	Fleet and Manners, 1976
Baker's yeast	22	NR	NR	NR	NR	NR	NR	98	0.26	97	3	Fleet and Manners, 1976
S. cerevisiae	20.4-24.5	NR	15-18	NR	2.4-6.2	NR	NR	NR	NR	18.3	14.2	Aguilar-Uscanga and François, 2003

[1]Protein = N x 5.5 as recommended for yeast (Reed and Nagodawithana, 1991)
[2]B1,3 = B1,3 glucans; B1,6 = B1,6 glucans; Carb = carbohydrate; N = nitrogen
[3]NR = not reported

Table 1.2. Nutritional composition of the intracellular components of *Saccharomyces cerevisiae*.

Type	Component[1]	DM (%)									Reference
		Total DM%	Dry weight	Protein[2]	Fat	Ash	Sugar	Carbohydrate	RNA	a-amino Nitrogen	
YE A	YE via HLY	15-20	95.2	62.5	0.1	9.5	2.9	NR	NR	NR	Podpora et al., 2016
YE B	YE via HLY	15-20	93.2	63.8	0.2	7.8	2.9	NR	NR	NR	Podpora et al., 2016
TUM 68 (Brewer's yeast)	YE via HLY	NR3	90.5	42.7	1	13.1	NR	31.3	5	1.7	Jacob et al., 2019
TUM 68 (Brewer's yeast)	YE via HLY	NR	90.6	42.9	1.1	13.3	NR	31.4	5	2.6	Jacob et al., 2019
TUM 68 (Brewer's yeast)	YE via ALY	NR	88.7	42.4	0.5	13.2	NR	28.8	4.9	4.5	Jacob et al., 2019
WYC (ethanol distillery)	WYC	NR	NR	37.6	0.5	4.6	NR	NR	9	NR	Yamada and Sgarbieri, 2005
PPC (ethanol distillery)	PPC	NR	NR	59.4	8.5	13.2	NR	NR	10.4	NR	Yamada and Sgarbieri, 2005
HLY (Brewer's yeast)	HLY	NR	NR	47.2	3.5	8.6	NR	21.5	7	NR	Cabellero-Cordoba and Sgarbieri, 2000
YPC (Brewer's yeast)	YPC	NR	NR	78.0	6.5	1.1	NR	9.1	2.3	NR	Cabellero-Cordoba and Sgarbieri, 2000
HWYC (Progut Rumen)	HWYC	35-40	95.0	34.0	1.8	25.0	NR	NR	NR	NR	Oeztuerk et al., 2016

[1]YE = yeast extract; HLY = hydrolyzed yeast; ALY = autolyzed yeast; WYC = whole yeast cell; PPC = phosphorylated yeast protein concentrate; YPC = yeast protein concentrate; HWYC = hydrolyzed whole yeast cell

[2]Protein = N x 5.5 as recommended for yeast (Reed and Nagodawithana, 1991)

[3]NR = not reported

Table 2.2. Summary of rumen fermentation, dairy cow performance, and animal health parameters in response to *Saccharomyces cerevisiae* supplementation. Specific differential responses are separated by dose or treatment if more than one is being compared.

Animal	Diet	Yeast Type	Dose	Response	Reference
Non-lactating cows	SLG[2], MH[2], and C[2] (48:42:10), grouped by degradability of NDF (low and high)	Levucell SC 10 ME	0.3 or 1.0 g/d of 1 x 10^{10} CFU[3]/g	<u>Dose 0.3 g/d</u> ↑[1] mean pH, total VFA[3] ↓[1] lactate, A: P[3] ≠[1] NH_3-N[3] <u>Dose 1.0 g/d</u> ↑ mean pH (0.3 < 1.0 g/d), total VFA, ↓ lactate (0.3 < 1.0 g/d), A: P (0.3 < 1.0 g/d) ≠ NH_3-N	Guedes et al., 2008
Non-lactating Holstein cows	TMR 50:50 (F:C)[3]	Y1[4] or Y2[4]	1.0 g (1 x 10^{10} CFU/d)	<u>Y1</u> ↑ acetate, A: P, ↓ propionate ≠ DMI[3], total VFA, lactate, NH_3-N, digestibility, CH_4[3] <u>Y2</u> ↑ propionate, AUC[3] (<5.8) ↓ acetate, A: P, mean pH, min pH, max pH ≠[1] DMI, total VFA, lactate, NH_3-N, digestibility, CH_4	Chung et al., 2011
Non-lactating cows	TMR (CS[2], AS[2], C)	Alltech MOS[4]	10.0 g/d	↑ serum titer[3] ≠ SPC[3], total serum Ig, packed cell volume (%), WBC[3], neutrophils (%), MNL[3] (%), eosinophils (%)	Franklin et al., 2005
Lactating dairy cows (meta-analysis)		YC[4]		↑ FCM (3.5%), ECM, milk fat (kg/d), milk protein (kg/d) ≠ milk fat (%), milk protein (%)	Poppy et al., 2011

Lactating dairy cows	CS, HCS[2], and H[2]	A-Max YC[4] or Celmanax (YC + HLY[4])	56 g/d (YC) or 28 g/d (YC + HLY)	A-Max ↑ total milk yield (kg/d), FCM[3] (3.5%), ECM[3], milk protein (%), milk fat (kg/d), milk protein kg/d) ≠ milk fat (%) Celmanax ↑ total milk yield (kg/d), FCM (3.5%), ECM, milk protein (%; Celmanax > A-Max), milk fat (kg/d), milk protein (kg/d; Celmanax > A-Max) ≠ milk fat (%)	Nocek et al., 2011
Lactating dairy cows	TMR ~50:50 (F: C)	CNCM I-1077; Levucell SC20	5.0 g/d (1×10^{10} CFU/d)	↑ mean rumen pH, min pH, max pH ↓ AUC (<5.6), AUC (<6.0)	Bach et al., 2007
Lactating dairy cows	TMR 50:50 (F: C)	Biosaf St. SC47	1.0 g (1×10^{10} CFU)/ 4 kg of DM	↑ DMI, total milk yield, FCM (4% per kg of DM), milk lactose (%), milk fat solids (g/d)	Moallem et al., 2008
Lactating Holstein cows	TMR 60:40 (F: C)	CNCM I-1077; Levucell SC20	0.5 g/d	↑ mean pH, min pH, max pH ↓ AUC (<5.6), AUC (<5.8), AUC (<6.0) ≠ DMI, total VFA, A: P, NH_3-N	Thrune et al., 2009
Lactating Holstein cows	TMR 50:50 (F: C)	Procreatin-7	2.0 g/d or 4.0 g/d	Dose 2.0 g/d ↑ acetate, DM digestibility, OM digestibility (2.0 > 4.0 g/d) ≠ DMI, mean pH, propionate, total VFA, A: P, total milk yield, milk components Dose 4.0 g/d ↑ acetate, DM digestibility, OM digestibility, NDF digestibility ≠ DMI, mean pH, propionate, total VFA, A: P, total milk yield, milk components	Ferraretto et al., 2012

Lactating dairy cows	TMR 70:30 (F: C)	Biosaf SC47	0.5 g/d or 5.0 g/d (1 x 10^{10} CFU/g of DM)	<u>Dose 0.5 g/d</u> ↑ mean pH ↓ lactate, NH_3-N ≠ total VFA, propionate, A: P <u>Dose 5.0 g/d</u> ↑ mean pH (0.5 < 5.0 g/d), total VFA, propionate ↓ lactate (0.5 < 5.0 g/d), NH_3-N (0.5 < 5.0 g/d) ≠ A: P	Pinloche et al., 2013
Lactating dairy cows		Yea-Sacc Farm Pak	25.0 g/d (1.07 x 10^8 CFU/g)	↑ total milk yield (kg/d), ECM, lactose, milk protein yield (kg/d) ↓ milk fat yield (kg/d)	Tristant and Moran, 2015
Lactating Holstein cows	TMR	Yea-Sacc YC	10.0 g/d	↑ DMI, CP digestibility, ADF digestibility ↓ lactate peak ≠ total milk yield, milk fat (%), milk protein (%), mean pH, lactate, total VFA, A: P, NH_3-N	Erasmus et al., 1992
Lactating Holstein cows	TMR 77:23 (F: C; wk[1] 1-6, HF[3]), TMR 49:51 (F: C; wk 7-10; HG[3])	Biomate	4 g/d (8 x 10^{10} CFU/d)	<u>HG</u> ↑ DMI, FCM (4%), total VFA, propionate ↓ AUC (<5.6), A: P ≠ total milk yield	AlZahal et al., 2014
Dairy cows	TMR 50:50 (F: C)	Proprietary strain A or B of live yeast	1 x 10^{10} CFU/d	<u>Strain A</u> ≠ digestibility, mean pH, total VFA, NH_3-N, A: P, CH_4, total milk yield, milk components <u>Strain B</u> ≠ digestibility, mean pH, total VFA, NH_3-N, A: P, CH_4, total milk yield, milk components	Bayat et al., 2015
Holstein heifers	100% forage (3 wk adaptation), increased grain	Diamond V XPC_{LS} YC	14 g/d	≠ DMI, mean pH, total VFA, A: P, lactate, NH_3-N	Moya et al., 2009

	load over 4 d until 10:90 (F: C) was reached and maintained for 10 d				
Holstein steers	5:95 (F: C)	NCYC 996 or MOS[4]	1.5 g/kg of DM	↑ mean pH ↓ NH_3-N, plasma LPS, plasma SAA ≠ DMI, digestibility, total VFA, A: P, min pH, max pH, AUC (<5.8), rumen LPS	Diaz et al., 2018
Beef heifers	TMR 50:50 (F: C)	Biomate ADY[4] or Biomate DDY[4]	4 g/d (1 x 10^{10} CFU/g of DM)	Biomate ADY ↑ mean pH, min pH ↓ pH duration (<5.8), pH duration (<5.6) ≠ DMI, max pH, total VFA, acetate, propionate, A: P, lactate, NH_3-N, digestibility, AUC (<5.8), AUC (<5.6) Biomate DDY ↑ mean pH , min pH ↓ pH duration (<5.8), pH duration (<5.6) ≠ DMI, max pH, total VFA, acetate, propionate, A: P, lactate, NH_3-N, digestibility, AUC (<5.8), AUC (<5.6)	Vyas et al., 2014
Lambs	TMR 40:60 (F: C; AC[3]), and 80:20 (F: C; RP[3])	CNCM I-1077; Levucell SC20	4 x 10^9 CFU/d	≠ mean pH, AUC (<5.6), total VFA, lactate, rumen LPS[3], plasma LPS[3], rumen SAA[3], plasma SAA[3]	Silberberg et al., 2013
Lambs	Hay (wk 1), hay plus concentrate where the proportion of concentrate was increased every 2 d from 25-50% (wk 2), and	Levucell SC20 (I-1077)	0.2 g/d (4 x 10^9 CFU/d)	≠ mean pH	Mosoni et al., 2007

	50:50 (hay: concentrate; wk 3)				
Rusitec (sheep inoculum)	60:40 (F: C)	Bio-Mos, YCW[4]	0.25 or 0.75 g/d	<u>Dose 0.25 g/d</u> ≠ mean pH, acetate, propionate, CH_4, NH_3-N, OM digestibility <u>Dose 0.75 g/d</u> ↑ propionate ≠ mean pH, acetate, CH_4, NH_3-N, OM digestibility	Oeztuerk et al., 2016
Batch culture (48 h; steer inoculum)	NS[2], or GC[2], or SS[2], or AH[2], or CBH[2]	Diamond V XP (YC[4]) or Saf Agri PMX70S BK (LY[4])	0.35 or 0.73 g/L	<u>YC 0.35 g/d</u> ↑ CH_4 (NS) ↓ mean pH (NS; AH; CBH), CH_4 (AH), A: P (NS) <u>YC 0.73 g/d</u> ↑ CH_4 (NS; 0.35 < 0.73) ↓ mean pH (NS; AH; CBH), A: P (NS; 0.35 < 0.73 g/d), A: P (GC; SS) <u>LY 0.35 g/d</u> ↑ mean pH (NS; AH), CH_4 (NS) ↓ A: P (NS; GC; SS) <u>LY 0.73 g/d</u> ↑ mean pH (NS; AH), CH_4 (NS; 0.35 < 0.73) ↓ A: P (NS; GC; SS)	Lynch and Martin, 2002
DF-CC[3]	10:90 (F: C) containing either CO[2] or B[2] as the C	CNCM I-1077; Levucell SC	2 x 10^7 CFU/g of DM	<u>CO</u> ↑ AUC (<6.0) ≠ digestibility, total VFA, individual VFA, A: P, NH_3-N <u>B</u>	Moya et al., 2018

		↓ NH_3-N, AUC (<6.0) ≠ digestibility, total VFA, individual VFA, A: P	
Ruminants (meta-analysis)	LY	↑ mean pH, total VFA, OM digestibility, DMI, total milk yield, ≠ lactate, milk fat (%), milk protein (%)	Desnoyers et al., 2009

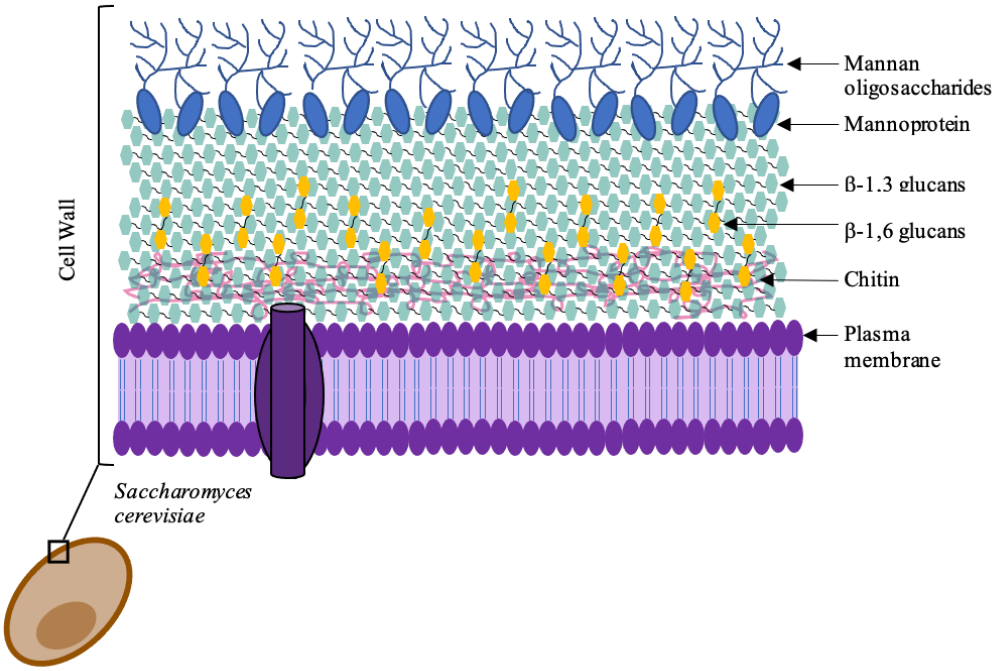
[1]↑ = increase; ↓ = decrease; ≠ = no change; wk = week

[2] SLG = silage; MH = meadow hay; C = concentrate; CS = corn silage; HCS = hay crop silage; AS = alfalfa silage; H = hay; NS = no substrate; GC= 0.4 g ground corn; SS = 0.4 g soluble starch; AH = 0.4 g alfalfa hay; CBH = 0.4 g coastal bermudagrass hay; CO = corn; B = barley

[3] F: C = forage: concentrate ratio; CFU = colony forming units; VFA = volatile fatty acids; A: P = acetate: propionate ratio; AUC = area under curve; DMI = dry matter intake; NH_3-N = ammonia nitrogen; FCM = fat-corrected milk; ECM = energy-corrected milk; HF = high forage; HG = high grain; AC = acidotic challenge; RP = resting period; serum titer = means of log_{10} reciprocals of the greatest dilution provided for neutralization of rotavirus; SPC = serum protein concentrate; WBC = white blood cell counts; MNL = mononuclear leukocytes; rumen LPS = rumen lipopolysaccharides; plasma LPS = blood plasma lipopolysaccharides; rumen SAA = rumen serum Amyloid A; plasma SAA = blood plasma serum Amyloid A; Rusitec= rumen simulation technique; DF-CC = dual-flow continuous culture

[4]Y1 = Levucell SC; Y2 = proprietary novel strain, Lallemand Animal Nutrition; ADY = active dry yeast; DDY= dead dry yeast; HWY = hydrolyzed whole yeast; LHWY = 3x less hydrolyzed whole yeast; YCW = yeast cell wall; YC = yeast culture; LY = live yeast; MOS= manno-oligosaccharides containing β-glucans and mannan; HLY = enzymatically hydrolyzed yeast

Figure 1.1 Structural components of the cell wall of *Saccharomyces cerevisiae*. Adapted from Santovito et al., 2018.



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Chapter 2: Impact of Dietary Yeast Extracts on Rumen Microbiota and Fermentation in Dual-Flow Continuous Culture

2.1 Abstract

Dietary yeast and yeast-derived extracts can influence the host rumen microbiota and subsequently the fermentation processes and metabolic products produced. The objective of this study was to evaluate six yeast extracts and their influence on the abundance and diversity of rumen bacteria, protozoa counts, digestibility, pH, and methane production. The current study was conducted as a 6 x 6 Latin square design using dual-flow continuous culture fermenters (n=6), including six 10-d periods each consisting of 7-d adaptation followed by 3-d collection period. Dietary treatments were included at 4% on a dry matter (DM) basis of the total diet, mixed with a total mixed ration and added to the fermenters twice daily in two equal portions (109 g DM total/fermenter/d). Treatments included 1) a Brewer's yeast extract with crude protein (CP) > 60% and a high degree of protein hydrolysis (BrE), 2) a blend of Brewer's yeast extract and Baker's peptone with CP > 65% and a mixture of high and low levels of protein hydrolysis and nucleotides (BrEPN), 3) a blend of Baker's yeast extract with CP > 50% and a mixture of high and medium levels of protein hydrolysis and nucleotides (BENH), 4) a blend of Baker's yeast peptone and yeast extract with CP > 65% and a mixture high and low levels of protein hydrolysis and nucleotides (BEPN), 5) a blend of Baker's yeast peptone, Brewer's yeast autolysate, and Baker's yeast extract with CP > 50% and medium protein hydrolysis (BEPBrA), and 6) a blend of Baker's yeast extracts with CP > 60% and a mixture of medium and low levels of protein hydrolysis and nucleotides (BENL). Fermenter and

overflow effluent samples were collected to determine nutrient digestibilities, rumen bacteria and protozoa enumeration, methane, fermenter pH, and bacterial DNA. Data were analyzed using the PROC MIXED procedure of SAS. Relative abundance and diversity of bacterial DNA was determined using R. Treatment did not affect protozoa and bacteria counts, digestibility, fermenter pH, methane concentration, bacterial relative abundance, or bacterial diversity. Spearman's correlation analysis revealed a greater number of correlations with fermenter pH parameters from supplementation with yeast treatment BrE, and treatment BENL had the greatest number of correlations with methane concentration. These results indicate that the inclusion of dietary yeast treatments with varied components, and processing do not differentially impact rumen bacterial relative abundance, diversity or fermentation patterns.

2.2 Introduction

Dietary yeast inclusion in ruminant diets has increased in popularity for its positive impact on animal performance, including milk production (Desnoyers et al., 2009), overall rumen fermentation (Erasmus et al., 1992), and feed efficiency (Moallem et al., 2009; Poppy et al., 2012). Higher rates of rumen fermentation and animal performance have largely been attributed to the stimulation in growth of select groups of rumen microbiota, mainly cellulolytic and lactate-utilizing bacteria species (Callaway and Martin, 1997; Chaucheyras-Durand et al., 2012; Ogunade et al., 2019). Supplementation of live yeast has been proposed to support the growth of cellulolytic bacteria by scavenging for oxygen, and creating a more favorable environment (Jouany and Morgavi, 2007). Moreover, yeast cell components are rich in nutrients (i.e., B vitamins, amino acids, peptides, organic acids) that

are used to promote the growth of both fiber-degrading (Wiedmeier et al., 1987), and lactate-utilizing bacteria (i.e., *Selenomonas ruminantium, Megasphaera elsdenii*; Callaway and Martin, 1997). Improvements of rumen fermentation parameters resulting from yeast supplementation has primarily been credited to increased fiber digestibility from the growth of cellulolytic bacteria (Chaucheyras-Durand et al, 2008), as well as shifts in the metabolic pathways of volatile fatty acids (VFA) from lactate-utilizing bacteria (Counotte et al., 1981), lowering the acetate: propionate ratio (AlZahal et al., 2014). Yeast inclusion has additionally increased ruminal pH of animals receiving concentrate-rich diets, thereby reducing the incidence of subacute ruminal acidosis (SARA; AlZahal et al., 2014). Moreover, animal health has been improved through inclusion of yeast cell wall components, such as manno-oligosaccharides and ß-glucans, which can reduce inflammatory responses from lipopolysaccharides (Diaz et al., 2018), enhance the innate immune system, and prevent the binding affinity of bacterial pathogens (Spring et al., 2015).

However, the impact of yeast supplementation in ruminant diets remains inconclusive due to the wide variability across yeast treatments and products. Differences in yeast products, origin, processing effect, components, strains, and dose yields inconsistences, especially coupled with differences in animal model used, diet composition, and experimental design. Yeast (i.e., Baker's yeast, Brewer's yeast) can differ in composition and its effect in the rumen (Pszczolkowski et al., 2016). Furthermore, yeast processing (i.e., autolysis, hydrolysis, degree of hydrolysis) can impact nutrient composition of yeast and rumen microbiota response (Mirzaei et al., 2015; Oeztuerk et al., 2016), while different components of yeast (i.e., cell wall, extracts,

etc.) also differ in their composition and their functionality in the rumen (Nocek et al., 2011; Oeztuerk et al., 2016; Diaz et al., 2018). Further investigation of the variable factors of yeast include strain (Newbold and Rode, 2006; Chung et al., 2011; Jurkovich et al., 2014), and dose (Ferraretto et al., 2012; Pinloche et al., 2013; Jiang et al., 2017) in ruminants.

Evaluation of the response differences in Baker's and Brewer's yeast and the processing effects of type and degree of hydrolysis of yeast in ruminants is limited. One study which evaluated two different commercialized forms of Baker's and Brewer's yeast in rumen fluid reported that Brewer's yeast decreased methane, acetate, and butyrate concentrations compared to Baker's yeast but did not influence propionate concentration (Pszczolkowski et al., 2016). This was likely due to the inhibitory effects of hop acids in Brewer's yeast (Pszczolkowski et al., 2016), as hop α- and ß-acids are secondary plant metabolites with inhibitory effects on gram positive bacteria in the rumen (Flythe, 2009). A second study examined the effect of a negative (no yeast) control compared with two different degrees of yeast hydrolysis (more or less) and yeast cell wall components on rumen fermentation using the rumen simulation technique (Rusitec; Oeztuerk et al., 2016). However, this study did not compare these treatments against one another and did not report the comparative differences between components or processing.

We hypothesized that the inclusion of dietary yeast extracts of different origin and processing would differentially impact the rumen microbiota and subsequent fermentation parameters. We further hypothesized that 1) the yeast extract treatment containing the greatest number of yeast cell components would have the greatest impact on the abundance and diversity of cellulolytic and lactate-utilizing bacteria from the diversity of growth-

promoting factors, and subsequently have improved fermenter pH and digestibility from increases in cellulolytic and lactate-utilizing bacteria, and 2) yeast extract treatments derived from Brewer's yeast would result in lower CH_4 concentrations. The objective of the current study was to evaluate the supplementation of six different dietary yeast extract treatments on rumen microbiota and fermentation, with supplements containing either one or a mixture of i) two different yeast origins (Baker's and Brewer's), ii) three different processing methods (low, medium, or high degree of protein hydrolysis), iii) three different components (extract, peptone, and autolysate), and iv) the absence or presence of yeast nucleotides. The specific aims were to i) evaluate relative abundance and diversity profiles of the rumen bacteria, principally focusing on cellulolytic and lactate-utilizing bacteria, and ii) assess rumen fermentation parameters, such as pH, digestibility, and methane concentrations.

2.3 Materials and Methods

2.3.1 Experimental Design and Diets

The experimental procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC # PROTO201900019) in accordance with The Public Health Service Policy on Humane Care and Use of Laboratory Animals. The study assessed six dietary treatments in a 6 x 6 Latin square design using *in vitro* dual-flow continuous culture fermenter systems. Each of the six 10-d periods included a 7-d adaptation period followed by 3-d of sample collection. Prior to the start of the experiment, corn silage-based TMR (Table 2.1) also fed to the lactating herd housed at the Paul R. Miller Research and Educational Center (South Burlington, VT) was collected, and

frozen at -20°C until further processing. Frozen feed was dipped in liquid nitrogen, mixed with dry ice, and ground to pass through a cooled 2mm screen (Wiley Mill, Thompson Scientific, Philadelphia, PA). Ground feed was then stored at -20°C until use. Each fermenter received 109 g DM of ground TMR (225 g as-fed calculated based on volume of fermenter and inclusion rates of previous successful continuous culture studies; Karnati et al., 2009; Wenner et al., 2017) separated into two equal feedings per d (0830 and 2030 h). In addition to the base TMR, each fermenter received one of six experimental treatments, which were mixed into the TMR prior to feeding. Yeast extract treatments are proprietary products supplied by Purina Animal Nutrition (Gray Summit, MO). Treatment descriptions are as follows: 1) a Brewer's yeast extract with CP > 60% with a high degree of protein hydrolysis (BrE**;** 3.6 g/d on a DM basis; calculated as 2% inclusion on an as-fed basis of the diet), 2) a blend of Brewer's yeast extract and Baker's peptone with CP > 65% with a mixture of high and low levels of protein hydrolysis and nucleotides (BrEPN**;** 3.6 g/d on a DM basis), 3) a blend of Baker's yeast extract with CP > 50% with a mixture of high and medium levels of protein hydrolysis and nucleotides (BENH**;** 3.5 g/d on a DM basis; calculated as 2% inclusion on an as-fed basis of the diet), 4) a blend of Baker's yeast peptone and yeast extract with CP > 65% with a mixture of high and low levels of protein hydrolysis and nucleotides (BEPN**;** 3.6 g/d on a DM basis), 5) a blend of Baker's yeast peptone, Brewer's yeast autolysate, and Baker's yeast extract with CP > 50% with medium levels of protein hydrolysis (BEPBrA**;** 3.5 g/d on a DM basis), and 6) a blend of Baker's yeast extracts with

CP > 60% with a mixture of medium and low levels of protein hydrolysis and nucleotides (BENL**;** 3.6 g/d on a DM basis). Each fermenter received each treatment (one treatment per period).

2.3.2 Continuous Culture Operation

Six dual-flow continuous culture fermenters (Electrolab Biotech, Tewkesbury, Gloucestershire, United Kingdom) with custom gravity-fed solid outflow spouts were used in the experiment. Systems were programmed to maintain a constant temperature at 39°C, and record temperature and pH every minute using indwelling sensors. A central agitator was programmed to continuously rotate internal paddles at 70 rpm followed by 200 rpm (for one min every 10 min) for the duration of the trial to ensure complete mixing. Mineral buffer as outlined by Weller and Pilgrim (1974), including 40 mg/dL urea addition, was added by peristaltic pumps to each fermenter. Clarified rumen fluid (rumen fluid that was strained through 4-layers of cheesecloth, centrifuged twice at 900 x *g* for 10 min to collect the supernatant, and autoclaved) was included in the buffer at 20% volume for the first 24 h of each period. Each fermenter was equipped with modified liquid outflow filters adapted from Karnati et al. (2009) attached to the end of pump-driven liquid outflow tubes for protozoa retention. Solid outflow, through gravity-fed outflow tubes, was combined with liquid outflow. The average fermenter volume was 2.98 L (± 0.16) and the average solid retention time and liquid dilution rate were 2.17% and 8.83%, respectively. Anaerobic conditions of each fermenter were maintained by continuously bubbling CO_2 into the buffer for at least 24 h before the start of each period and continued for the duration of the period.

At the beginning of each period, rumen fluid was collected from the ventral, dorsal and central portions of the rumen (Dillard et al., 2018) of three fistulated lactating Holstein cows two hours post-feeding at 0930 h (University of Vermont's Paul R. Miller Research and Educational Center, South Burlington, Vermont). The fluid was immediately sifted through a strainer to remove larger feed particles, and placed into a 5-gallon bucket with heating jacket (Powerblanket, Salt Lake City, UT) set to 39°C. Within 30 min from the start of each collection, the rumen inoculum from each cow was combined, mixed, and evenly distributed to the pre-warmed fermenters until the fluid surpassed the gravity outflow spout.

2.3.3 Sample Collection

Aliquots of rumen fluid were collected prior to fermenter inoculation. The rumen fluid aliquots were strained through 4-layers of cheesecloth, and blended using a Rocket Blender (Bella, Montréal, Canada) for 60 s to dislodge microbial cells from feed particles as described in Lascano et al. (2009). A subsample of blended rumen fluid was then fixed in 37% formaldehyde (Dehority et al., 1993) at 25% of the sample volume for later determination of protozoal counts. To remove protozoa from each sample, a second subsample was strained through a 50 μm nylon bag (Ankom Technology, Macedon, NY) and then fixed in 37% formaldehyde at 25% of the sample volume for enumeration of rumen bacteria as per Lascano et al. (2009). This process was also repeated on d 3, 6, and 9 using fermenter contents aspirated directly from each fermenter vessel via a 60 mL syringe adapted with peristaltic tubing. On d 7, overflow effluent and filter effluent bottles

were placed on ice to halt microbial fermentation. At the beginning of each sampling day (d 8, 9, and 10), methane measurements were recorded in triplicate prior to each feeding. Methane (% volume) of the gas headspace was measured using the Sewerin Multitec 545 gas analyzer (Sewerin, Gütersloh, Germany) with an attached coolant coil (Sewerin, Gütersloh, Germany) by inserting the gas probe directly into the port of each fermenter. Methane concentration (mg/dL) was calculated as (% volume of methane x 554)/10 and taken as the mean within treatment and sampling days within each period. At 1000 h on d 8, 9 and 10, total 24-h effluent of each fermenter was collected, weighed, and subsamples were taken from each fermenter for further analysis. Subsamples for microbial analysis were immediately processed. To isolate bacteria for DNA extraction and 16S rRNA sequencing, bacterial pellets were harvested from effluent samples as outlined by Del Bianco Benedeti et al. (2015), except that 4-layers of cheesecloth were used. Each bacterial pellet from each of the sampling days was pooled together in equal proportion within fermenter within period and frozen at -20°C until further analysis. To determine DM disappearance and apparent NDF, ADF, OM, and DM digestibility, representative 1.5 L subsamples of effluent from each fermenter were also collected at 1000 h on d 8, 9, and 10 and frozen at -20°C until further analysis.

2.3.4 Enumeration of Protozoa and Bacteria

Protozoa samples (d 0, 3, 6, and 9) of each period were stained according to Dehority et al. (1993). Each sample was placed into a Sedgwick Rafter Cell (Hausser Scientific, Horsham, PA) and counted microscopically at 100x magnification using the Fisher Micromaster (Fisher Scientific, Waltham, MA).

Bacterial samples were diluted with ddH_2O at a 2000:1 ratio and then stained with a 1X solution of SYBR® Green I nucleic acid gel stain (Invitrogen, Carlsbad, CA) using 50 µL of stain for every 500 µL of sample used. Staining was performed no more than 30 min prior to flow cytometry analysis. Bacterial samples were enumerated via flow cytometry (Flow Cytometry and Cell Sorting Facility, Learner College of Medicine, University of Vermont) using the Cytek Aurora 4 laser through the SpectroFlow software package v2.2.0 (Cytek Biosciences, Fremont, CA). Excitation of SYBR® Green I nucleic acid gel stain was measured on a 488 nm laser and its emission was collected on a B6 channel (BP 525/17).

2.3.5 Determination of Nutrient Digestibility

DM was determined by oven drying subsamples at 65°C for 48 h and calculated by weight difference. Dried samples were pooled within fermenter within period and analyzed for wet chemistry (Dairy One, Ithaca, NY). Apparent nutrient digestibilities (DM, OM, NDF, and ADF) were subsequently calculated as per Soder et al. (2016).

2.3.6 Microbial DNA Extraction of the 16S rRNA Gene

Enriched lyophilized bacterial pellets were pooled within period within fermenter and were used to extract bacterial DNA for 16S rRNA amplification and sequencing. Each bacterial pellet was ground to a powder-like consistency using 5 mm grinding balls (Ops Diagnostics, Lebanon, NJ) on the TissueLyser II (Retsch, Newtown, PA) at 20 hz for 5 min. Of the ground samples, 10 mg sample was rehydrated in PBS and centrifuged (Sorvall

Legend Micro 21R, ThermoFisher Scientific, Waltham, MA) at 5,000 x *g* for 10 min to obtain the bacterial pellet. Bacterial DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The purity of extracted DNA was verified using a Nanodrop 2000c Spectrophotometer (ThermoFisher Scientific, Waltham, MA) and quantified using a Qubit® 3.0 Fluorometer (ThermoFisher Scientific, Waltham, MA). The DNA libraries were created by The University of Michigan Host Microbiome Core (University of Michigan, Ann Arbor, MI) as previously described by Koenigsknecht et al. (2015). PCR amplification was performed using barcoded dual-index primers that target the V4 region of the 16S rRNA gene as reported by Kozich et al. (2013). Resulting PCR amplicons, library preparation, and sequencing were performed similarly to Seekatz at al. (2015) except that the final library concentration load contained 5.5 pM and 15% PhiX. Sequencing reagents were prepared according to Kozich et al. (2013), containing custom read 1, read 2 and index primers that were added to the reagent cartridge. The generation of FASTQ files with paired end reads were used for data analysis.

2.3.7 Statistical Analyses

Data was analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC) according to the following model:

$$Y_{ijk} = \mu + f_i + P_j + T_k + e_{ijk},$$

where Y_{ijk} is the observed dependent variable, μ is the overall population mean, f_i is the random effect of the ith fermenter (i = 1, 2, 3, 4, 5, 6), P_j is the fixed effect of the jth period (j= 1, 2, 3, 4, 5, 6), T_k is the fixed effect of kth treatment (k = 1, 2, 3, 4, 5, 6), and e_{ijk} is the residual error. The Kenward Roger degrees of freedom correction was applied to all

statistical analyses and all values reported are shown as least square means. In period 4, treatments were analyzed without treatment BEPN (resulting n = 5 treatment) due to culture termination. Log transformation was applied to protozoa and bacteria data to achieve normal distribution prior to analysis. For protozoa and bacteria enumeration, the random statement was used with fermenter within period as the subject. Significance was declared at $P \leq 0.05$.

2.3.8 Bioinformatics Analyses of 16S rRNA gene sequences

Raw sequence data are available via the Sequence Read Archive (SRA) and can be accessed from the NCBI BioProject accession number PRJNA673854. Paired-end sequences from each sample were used to generate operational taxonomical units (OTU) via the mothurPipeline Github repository (https://github.com/wclose/mothurPipeline). This pipeline utilized the Snakemake-based implementation from MOTHUR with count file subsampling set to 1000 reads per sample. Generated OTU's were taxonomically aligned to the SILVA v132 reference database (Quast et al., 2012) and were subsequently used for the creation of a phyloseq object via the package 'phyloseq' (version 1.32.0; McMurdie and Holmes, 2013) in R version 4.0.2.

Prior to analysis, OTU's with fewer than 3 sequences were removed (Popova et al., 2019). Alpha diversity metrics, including Richness (Chao1), Shannon's diversity, Fisher's alpha, and Pielou's Evenness were calculated using the package microbiome (Lahti et al., 2020) and were analyzed using two-way ANOVA for the effects of treatment and period via R. Treatment and period ordination were visualized by non-metric multidimensional scaling (NMDS) using Bray Curtis dissimilarity following the Hellinger transformation using the package phyloseq. The betadisper function of the vegan package was used to

corroborate the homogeneity condition of group dispersions before running PERMANOVA analysis using the Adonis function of the vegan package. The relative abundance of bacteria between treatments was determined at the genus level and aggregated by the top 10 most abundant taxa prior to performing ANOVA. Dependent variables and their residuals were evaluated for normal distribution via Shapiro-Wilk normality tests and Q-Q plots. The following are dependent variables that did not conform to normal distribution and were transformed: methane (mg/dL), pH range, AUC < 5.6 and 5.5, Shannon's diversity, Pielou's evenness, apparent DM and OM digestibility via ordered quantile normalization (i.e., ORQ; Peterson and Cavanaugh, 2019). Correlation analysis utilized Spearman's rank via the cor function of stats to generate matrices, the significance threshold was set to $P < 0.05$, and was visualized using the corrplot function from the corrplot package.

2.4 Results

2.4.1 Fermenter pH

Fermenter pH mean, minimum, maximum, and range were not different between treatments (Table 2.3). Furthermore, analysis of time spent under pH thresholds calculated as the area under the curve (AUC) of pH thresholds <5.5 (min/d), <5.6 (min/d), and <5.8 (min/d) were not different across treatments.

2.4.2 Protozoa and Bacteria Enumeration

Protozoa counts were not affected by treatment (Table 2.4). The day of sampling for which protozoa were collected showed decreased protozoa counts as each experimental

period progressed ($P < 0.0001$). Protozoa counts on d 9 were lower (3.34×10^4) compared to d 3 (4.84×10^4; $P < 0.01$) and d 6 (4.65×10^4; $P < 0.01$), while there were no differences between d 3 and d 6.

Similarly to protozoa counts, bacteria counts were not affected by treatment (Table 2.4) and no changes in total bacteria counts from yeast extract supplementation were observed. However, unlike protozoa counts, which decreased throughout the course of each 10-d period, bacteria counts remained stable throughout the length of each experimental period.

2.4.3 Methane

Methane concentrations (% volume) of the gas headspace were not affected by treatments (Table 2.3).

2.4.4 Relative Abundance and Diversity of Bacteria

Bacterial diversity was not different between treatment groups (Figure 2.1). Furthermore, alpha diversity metrics, including Pielou's evenness, Chao1 richness, Shannon's diversity, and Fisher's alpha of bacteria, were not different between treatments (Figure 2.2).

Relative abundance of bacteria at the genus level was not different between treatments (Figure 2.3). The relative abundance of lactate-utilizing genera *Selenomonas* and *Megasphaera* comprised 1.71-2.58% ($P = 0.97$) and 0.35-1.26% ($P = 0.45$) of the rumen bacteria among yeast extract treatments, respectively (Table 2.6).

2.4.5 Nutrient Digestibility

The mean apparent digestibility of DM from each treatment ranged between 60.6-72.6% and was not affected by treatment (Table 2.5). Likewise, apparent digestibility of OM (mean: 65.1-77.1%), NDF (mean: 46.4-59.5%), and ADF (mean: 53.2-72.1%) were not different between treatments (Table 2.5).

2.4.6 Yeast Extract Treatment Correlations on Fermenter pH and Methane

Correlation analysis using Spearman's rank revealed associations between rumen bacterial genera and pH and methane when provided dietary yeast treatments BrE and BENL. The treatment BrE had the highest number of associations, relating to fermenter pH, including minimum, maximum, mean, range, and the area under 5.5, 5.6, and 5.8 (Figure 2.4). With BrE treatment, fermenter pH minimum was negatively correlated with the unclassified genera (containing multiple OTU's) *Bacteroidales* (r = -0.89; $P < 0.05$) and *Bacteroidetes* (r = -0.88; $P < 0.05$). Moreover, pH maximum was negatively associated with the unclassified genera of *Clostridiales* (r = -0.82; $P < 0.05$). The pH mean was negatively associated with the unclassified genera of *Bacteria* (r = -0.88; $P < 0.05$) and *Bacteroidetes* (r = -0.92; $P < 0.05$), while the pH range was negatively associated with the unclassified genera of *Clostridiales* (r = -0.90; $P < 0.05$). Additionally, pH AUC < 5.5, 5.6, and 5.8 were all positively associated with the unclassified genera of *Bacteroidetes* (r = 0.86, 0.88, 0.94; $P < 0.05$, respectively) while only AUC <5.5 was positively associated with the unclassified genera of *Bacteria* (r = 0.83; $P < 0.05$).

Supplementation with yeast extract treatment BENL resulted in the greatest number of correlations on methane concentration (Figure 2.5). A positive correlation between

methane concentration and the genus *Prevotella* (r = 0.90; $P < 0.05$) was determined. Furthermore, negative correlations were observed between methane concentrations and the unclassified bacterial genera *Bacteria* (r = -0.93; $P < 0.01$), *Bacteroidetes* (r = -0.84; $P < 0.05$), *Clostridiales* (r = -0.94; $P < 0.01$), as well as diversity indices, including Shannon's diversity, Chao1 richness, and Fisher's alpha (r = -0.85, -0.85, -0.84, $P < 0.05$; respectively).

2.5 Discussion

This study evaluated the effect of six yeast extract supplements harvested from different sources having undergone different processing methods on the rumen microbiota and their functions. Our study evaluated yeast extracts from Brewer's or Baker's yeast or a combination thereof. The yeast extracts were processed differently to yield different forms of yeast extracts, including peptones, and autolysates. Furthermore, processing methods were altered to achieve different degrees of protein hydrolysis. The amount of CP differed between these treatments as well as the inclusion of nucleotides from the selected yeast. To our knowledge, this is the first study to examine these differences between yeast extracts on the rumen microbiota and fermentation parameters.

2.5.1 *In Vitro* Assessment of Dietary Yeast Extract Supplementation on Rumen Protozoa and Bacteria

One of the most crucial aspects of continuous culture operation is the maintenance of microbial populations and creation of an environment that allows for microbial functionality to reflect an *in vivo* response. The protozoa counts from our study (range: 3.7-4.8 x 10^4/mL) were greater than those presented by Karnati et al. (2009; range: 1.7-2.3 x

10^4/mL) and Wenner et al. (2017; range: 0.7-1.4 x 10^4/mL) using similar filters modified for protozoa retention. Previous studies have reported that yeast supplementation increases rumen protozoa counts (Kowalik et al., 2012; Shen et al., 2018; Chaucheyras-Durand et al., 2019). Conversely, bacterial counts measured in our study were lower than those observed in a previous culture study (9.35 x 10^5/mL versus 4.78 x 10^8/mL, Newbold et al., 1998). It is likely that the method used for enumeration of bacteria played a role in the differences observed. Newbold et al. (1998) determined total viable bacteria counts by cultivating cellulolytic bacteria on cellulose agar as well as microscopically counting bacteria as opposed to flow cytometry used in the current study. Furthermore, rumen bacteria counts in the current study could have been lower due to the rumen fluid collection method. Direct rumen fluid extraction from the fermenters was accomplished through aspiration using peristaltic tubing, which limited the amount of feed particles within each sample. The majority of rumen bacteria (70-80%) reside on the surface of feed particles while the remainder (20-30%) are free-floating in the liquid fraction (Miron et al., 2001). The limited amount of feed particles and their associated bacteria in our samples likely contributed to the lower bacteria counts observed. Furthermore, bacteria counts were not different between experimental treatments in the current study. Few studies determined that yeast supplementation can increase total bacteria counts (Newbold et al., 1995; Newbold et al., 1996; Lascano et al., 2009). The study conducted by Newbold et al. (1996) compared the total viable bacterial counts *in vitro* of Yea-Sacc (Alltech, Inc., Nicholasville, KY) and four different strains of *Saccharomyces cerevisiae* yeast cultures. This study showed that Yea-Sacc and two *Saccharomyces cerevisiae* yeast culture strains increased bacteria counts compared to the other two *Saccharomyces cerevisiae* yeast culture strains

and the control (Newbold et al., 1996). The study suggests that there are differences in the efficacy of yeast supplementation on rumen bacteria.

When evaluating six different novel yeast extract supplements on the relative abundance of rumen bacteria in the current study, we observed no treatment differences of yeast extract supplementation on the relative bacterial abundance in continuous culture. A study by Mohammed et al. (2017), who supplemented active and killed dried yeast also did not find any differences on rumen bacterial relative abundance when supplementing either yeast treatment. Our study, observed that the unclassified genera of *Lachnospiraceae* had the greatest abundance among the treatment groups, followed by *Prevotella* and the unclassified genera of *Ruminococcaceae.* These rumen bacteria have been reported to be in the highest relative abundance in numerous studies (Schären et al., 2017; Freetly et al., 2020; Jose et al., 2020; Welty et al., 2019), which is in line with our study. Dietary yeast supplementation has been reported to alter the relative abundance of cellulolytic and lactate-utilizing bacteria in the rumen (Pinloche et al., 2013; Jiang et al., 2017). However, results have not always been consistent regarding the changes in cellulolytic and lactate-utilizing bacteria. A study from AlZahal et al. (2014), observed the addition of yeast did not alter the relative abundance of the lactate-utilizing bacterium *Selenomonas,* which has been supported by Welty et al. (2019) who supplemented a yeast culture in their experiment. These results are in line with our current study, in which no changes in the relative abundance of lactate-utilizing bacteria between yeast extract treatments were observed. However, lactate-utilizing bacteria have been shown to take longer to replicate in the rumen, and adaptation to changes in diet can take several weeks (Monterio and Faciola, 2020); hence, the short period length of continuous culture trials (i.e., 10 days)

may limit the ability to promote these shifts *in vitro*. Many studies have elucidated changes in relative abundance of rumen bacteria after yeast supplementation against a negative control, but very few have evaluated the changes among different yeast types. We originally hypothesized that there would be changes in relative abundance of bacteria in response to the Brewer's yeast extract treatments (BrE, BrEPN, BEPBrA) in the current study due to the inhibitory effects of hop acids on gram-positive bacteria. Although we did not observe these results, further evaluation of these treatments at a higher dose could be useful to further characterize their impact on gram-positive bacteria.

Following the evaluation of the abundance of rumen bacterial genera among treatments, we evaluated α-diversity, which measures the diversity of bacteria within each sample. Metrics used to evaluate α-diversity included Chao1, an estimator of species richness based on OTU (Chao, 1984), Shannon and Fisher's alpha, a measure to indicate species abundance and diversity (Shannon, 1948; Fisher et al., 1943), and Pielou's evenness, a measure of species evenness (Smith and Wilson, 1996). Within our study, we observed that within-sample (α) bacterial diversity was not different among any of the yeast extract treatments. Studies that evaluated within-sample (α) bacterial diversity from dietary yeast supplementation have only been reported by comparison of yeast with a negative control. Meller et al. (2019) supplemented *Saccharomyces cerevisiae* live yeast culture (5.0% DM/basis; YeaSacc^{1026}, Alltech Inc., Nicholasville, KY) in the diet of lactating Jersey cows and found that Shannon diversity and richness were not different as a result of yeast compared to a negative control. Additionally, Pinloche et al. (2013) who supplemented live yeast (BIOSAF SC 47, Lesaffre Feed Additives, France) at 0, 0.5 or 5 g/d compared to a control and found no differences in Shannon diversity among treatment

groups. Diet appears to be the primary driver of diversity shifts in the rumen (Henderson et al., 2015). Our study as well as the aforementioned studies, likely did not see bacterial diversity shifts because basal diet was uniform amongst all treatment groups.

The β-diversity metric measures the differences in diversity between samples. Our current study evaluated β-diversity among treatments using Bray-Curtis dissimilarity (Bray and Curtis, 1957), which evaluates the diversity of the distance of dissimilarity between each sample. Similar to within-sample α-diversity, between-sample β-diversity was not different between experimental treatments in the current study. Other studies that have evaluated yeast supplementation against a negative control on β-diversity and found no differences due to the addition of yeast (Meller et al., 2019; AlZahal et al., 2017; Chaucheyras-Durand et al., 2019). These results, as well as those of the current study, could be a result of a uniform basal diet provided within all treatment groups.

2.5.2 Impact of Dietary Yeast Extracts on Rumen Performance Metrics of pH, Methane, and Digestibility

The impact of yeast supplementation on digestibility has been inconsistent (Miller-Webster et al., 2002; Chung et al., 2011; Diaz et al., 2018), resulting from differences in the composition of the base diet and microbial composition of the inoculum used. An important mode of action of yeast supplementation appears to be the resulting increase in NDF digestibility, which is commonly observed with increases in the number of cellulolytic species (Pinloche et al., 2013). In our study, we did not observe any differences between yeast extract treatments on bacterial relative abundance in any bacterial genera, and in line with this, we did not observe changes in NDF digestibility. Our research did not

include a negative control and utilized a high concentrate diet (50: 50 forage: concentrate ratio), which lowers the reliance of nutrient liberation by cellulolytic bacteria from fibrous carbohydrates. A recent study from Diaz et al. (2018) observed no differences in apparent DM and NDF digestibility when supplementing cannulated Holstein steers fed a 95% concentrate diet with either 1.5 g/kg of DM of live yeast (*Saccharomyces cerevisiae*), manno-oligosaccharides, or a control. A meta-analysis conducted by Desnoyers et al. (2009) highlighted that yeast supplementation often increases OM digestibility, but that these positive effects of yeast are diminished with an increasing proportion of concentrate in the diet. This is likely the reason that our study did not observe positive effects of yeast extract supplementation on DM and OM digestibility. This is further supported by Chung et al. (2011) who fed a 50: 50 (forage: concentrate) basal diet to Holstein dairy cows supplemented with two different active dry yeast strains of *Saccharomyces cerevisiae* and observed no differences in DM, OM, NDF, or ADF digestibilities compared to a negative control.

Other metrics, such as fermenter pH, were in line with a higher concentrate diet. Our study observed pH values commonly associated with increased risk of SARA. A similar study that supplemented a high concentrate diet with live yeast (2×10^7 CFU/g of diet, *Saccharomyces cerevisiae,* CNCM I-1077 Levucell, Lallemand SAS, Blagnac, France) added to dual-flow continuous culture fermenters found that fermenters reached the minimum pH limit (5.5) daily; however, these declines in pH were circumvented by automatic addition of NaOH (Moya et al., 2018), which was not used in the current study. This study was also different from our study in that the authors observed that the addition of live yeast with barley grain reduced the AUC (6.0) and increased the time until minimum

pH was reached (Moya et al., 2018). Conversely, Chung et al. (2011) concluded that a novel active dry yeast strain (Lallemand Animal Nutrition, Montréal, QC, Canada, 1 x 10^{10} CFU/head/day) fed to Holstein dairy cows lowered pH parameters and increased AUC (5.8) compared to a control and the Levucell SC (Lallemand Animal Nutrition, Montréal, QC, Canada, 1 x 10^{10} CFU/d) treatments. These studies suggest that the components and variability within yeast can influence rumen pH both positively and negatively. None of the supplements examined in the current trial elicited a differential response in fermenter pH, and further comparison of these treatments against a control would provide further insight.

Methane concentration observed in the current study also supports that microbial activity and nutrient capture were not affected by dietary yeast extract treatments. Studies that have evaluated the difference of two strains of *Saccharomyces cerevisiae* yeast on methane concentrations in dairy cows (Chung et al., 2011; Bayat et al., 2015) found no differences between yeast treatments. This was also observed by McGinn et al. (2004) who supplemented two different yeast products (Levucell SC vs. Procreatin-7 yeast) in Holstein steers and by Lynch and Martin (2002) who compared the difference of live yeast and yeast culture *in vitro,* both of which showed no differences in methane concentrations between yeast treatments. Our study corroborates these findings, suggesting that yeast strain or products do not differentially impact methane concentrations in ruminants. Further evaluation of the yeast extract treatments in the current study against a control would be necessary to establish the efficacy of their inclusion and determine if these yeast extract treatments increase methane concentrations, which has previously been observed (Lynch and Martin, 2002).

2.5.3 Correlations on Fermenter pH and Methane from Yeast Extract Supplementation

While individual metrics were not affected by treatment, various correlations were identified within unique treatments which can help guide further studies.

Supplementation with BrE had the greatest number of correlations associated with fermenter pH parameters. Although there were no direct differences observed on fermenter pH among any of the yeast extract treatments, further evaluation of BrE treatment could provide a greater understanding on why these correlations occurred and the reason for why there were more correlations for BrE than with the other yeast extract supplements. Furthermore, the addition of BENL showed a greater number of correlations associated with methane concentrations within the fermenters. With current research interest investigating dietary yeast extract supplementation to mitigate methane, further investigation of BENL and its association with methane could contribute to the body of knowledge regarding yeast components and processing and their potential on methane mitigation strategies.

2.6 Conclusions

We aimed to comparatively evaluate six yeast extract treatments with different components and processing effect on *in vitro* rumen protozoa, bacterial abundance and diversity, and subsequent fermentation parameters including pH, digestibility, and methane. We hypothesized that yeast extracts comprising of Brewer's yeast (BrE, BrEPN, and BEPBrA) would negatively impact gram positive bacteria and lower methane concentration in the fermenters, while diverse components within BEPBrA would

stimulate the growth of cellulolytic and lactate-utilizing bacteria, thus increasing digestibility and fermenter pH parameters. The addition of either yeast extract (i.e., BrE, BrEPN, BENH, BEPN, BEPBrA, and BENL) did not differentially affect rumen protozoa and bacteria counts, bacterial relative abundance, bacterial diversity, or rumen fermentation parameters of digestibility, fermenter pH, and methane concentrations. Furthermore, correlation analysis identified the yeast extract treatment BrE to have the greatest number of correlations on fermenter pH parameters, while treatment BENL resulted various correlations related to methane output. Future research should include a dose-response study to determine the efficacy of these yeast extracts, an *in vivo* study to determine the impact of supplementation on rumen protozoa, and milk production parameters, as well as a greater evaluation of treatment BrE on rumen pH and BENL on rumen methane concentration.

2.7 Acknowledgements

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Table 2.1. Chemical composition) of the total diet (TMR + yeast extract treatments) within continuous culture fermenters.

	Treatment[1]					
Item	BrE	BrEPN	BENH	BEPN	BEPBrA	BENL
Composition, % of DM						
OM	92.4	92.4	92.3	92.3	92.6	92.2
CP	20.5	20.7	20.2	20.7	20.2	20.4
NDF	26.7	26.7	26.6	26.7	26.7	26.6
ADF	17.1	17.1	17.1	17.1	17.1	17.1
Lignin	2.9	2.9	2.9	2.9	2.9	2.9
NFC_2	40.5	40.4	40.8	40.3	41.0	40.5
Ash	7.6	7.6	7.7	7.7	7.4	7.8
Starch	27.4	27.4	27.3	27.3	27.3	27.3
Ether extract	4.7	4.7	4.7	4.7	4.7	4.7
$NE_{M,}$ Mcal/Kg	1.8	1.8	1.8	1.8	1.8	1.8
$NE_{G,}$ Mcal/Kg	1.2	1.2	1.2	1.2	1.2	1.2
$NE_{L,}$ Mcal/Kg	1.8	1.8	1.8	1.8	1.8	1.8
Ca	0.74	0.74	0.74	0.74	0.74	0.74
P	0.54	0.52	0.52	0.52	0.51	0.53
Mg	0.40	0.39	0.39	0.39	0.39	0.39
K	1.4	1.3	1.4	1.4	1.3	1.4
Na	0.76	0.75	0.77	0.77	0.73	0.80
S	0.38	0.37	0.39	0.40	0.35	0.40
Cu, ppm	34.4	34.4	34.3	34.3	34.6	34.3
Chloride Ion	0.58	0.58	0.58	0.57	0.57	0.57
DCAD, mEq/kg	272	270	282	276	271	286

[1] BrE = Brewer's yeast extract > 60% CP with a high degree of protein hydrolysis
BrEPN = Blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides
BENH = Blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides
BEPN = Blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides
BEPBrA = Blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with a mixture of medium levels of protein hydrolysis
BENL = Blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides
[2] NFC = 100 − (%NDF + %CP + %EE + %Ash).

Table 2.2. Chemical composition (% of DM) of yeast extract treatments provided in continuous culture fermenters.

	Treatment[1]						
Item	BrE	BrEPN	BENH	BEPN	BEPBrA	BENL	SEM
OM	86.5	86.1	82.5	83.4	90.0	80.8	1.3
CP	73.4	75.8	62.5	74.3	60.9	68.0	2.6
ADF	2.7	2.6	1.1	3.6	1.2	1.4	0.4
NDF	1.2	1.7	0.6	2.6	1.6	1.0	0.3
Ether extract	0.2	0.1	0.1	0.1	0.1	0.1	0.02

[1] BrE = Brewer's yeast extract > 60% CP with high degree of protein hydrolysis
BrEPN = Blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides
BENH = Blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides
BEPN = Blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides
BEPBrA = Blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis
BENL = Blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides

Table 2.3. pH parameters and methane concentrations for continuous culture fermenters supplemented with yeast extract treatments. Values are expressed as the mean.

	Treatment[1]							*P*-value[2]	
Variable	BrE	BrEPN	BENH	BEPN	BEPBrA	BENL	SEM	T	P
Fermenter pH									
Mean	5.8	5.8	5.8	5.6	5.7	5.8	0.04	0.36	0.01
Minimum	5.0	5.0	4.9	4.9	4.9	5.1	0.03	0.39	0.06
Maximum	6.8	6.7	6.7	6.6	6.7	6.6	0.03	0.86	0.19
Range	1.8	1.7	1.8	1.8	1.9	1.6	0.04	0.49	0.52
pH < 5.8									
Duration (min/d)	615	653	714	933	920	681	56.52	0.31	0.01
AUC[3], pH x min/d	240	159	194	293	313	180	25.72	0.49	0.06
pH < 5.6									
Duration (min/d)	297	469	476	471	645	358	48.75	0.48	0.03
AUC, pH x min/d	132	57	71	145	160	76	17.94	0.46	0.11
pH < 5.5									
Duration (min/d)	168	332	352	349	493	231	45.86	0.48	0.04
AUC, pH x min/d	91	32	38	93	106	43	13.45	0.44	0.14
CH_4, mg/dL	28.7	52.9	36.1	15.0	59.7	19.5	7.3	0.44	0.34

[1] BrE = Brewer's yeast extract > 60% CP with a high degree of protein hydrolysis
BrEPN = Blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides
BENH = Blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides
BEPN = Blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides
BEPBrA = Blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis
BENL = Blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides
[2] *P*-values are reported to show the main effects of treatment (T) and period (P).
[3] AUC = area under the curve

Table 2.4. Counts of protozoa and bacteria populations of yeast extract treatments within continuous culture fermenters. Values are expressed as the mean.

	Treatment[1]							*P*-value[2]			
Variable	BrE	BrEPN	BENH	BEPN	BEPBrA	BENL	SEM	T	P	D	T x D
Protozoa (cells x 10^4/mL)								0.59	<0.001	<0.001	0.62
Day 3	5.3	4.3	5.1	5.1	5.5	4.0	0.23				
Day 6	4.8	4.4	5.4	4.6	5.1	3.8	0.23				
Day 9	3.4	3.2	4.0	3.2	3.0	3.5	0.15				
Bacteria (cells x 10^5/mL)								0.44	<0.001	0.20	0.30
Day 3	8.4	8.5	9.3	13.9	8.8	7.7	0.92				
Day 6	7.2	7.4	8.6	7.8	9.0	8.3	0.29				
Day 9	7.1	7.6	8.7	6.8	9.8	9.2	0.49				

[1] BrE = Brewer's yeast extract > 60% CP with a high degree of protein hydrolysis

BrEPN = Blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels protein hydrolysis and nucleotides

BENH = Blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides

BEPN = Blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides

BEPBrA = Blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis

BENL = Blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides

[2] *P*-values are reported to show the main effects of treatment (T), period (P), day (D), and the treatment by day interaction (T x D).

Table 2.5. Apparent nutrient digestibilities of a TMR diet and yeast extract treatments within continuous culture fermenters. Values are expressed as the mean.

	Treatment[1]							*P*-value[2]	
Variable	BrE	BrEPN	BENH	BEPN	BEPBrA	BENL	SEM	T	P
Apparent digestibility									
DM, %	60.6	64.8	68.5	72.6	64.9	62.5	1.8	0.18	0.0002
OM, %	65.1	69.4	73.2	77.1	70.0	68.5	1.7	0.16	0.0002
NDF, %	46.4	57.1	49.6	58.5	59.5	56.0	2.1	0.56	0.12
ADF, %	53.2	65.4	58.0	72.1	70.7	67.4	3.0	0.20	0.02

[1] BrE = Brewer's yeast extract > 60% CP with high degree of protein hydrolysis

BrEPN = Blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels protein hydrolysis and nucleotides

BENH = Blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides

BEPN = Blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides

BEPBrA = Blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis

BENL = Blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides

[2] *P*-values are reported to show the main effects of treatment (T) and period (P).

Table 2.6. Relative abundance (%) of lactate-utilizing bacteria genera *Selenomonas* and *Megasphaera* of yeast extract treatments within continuous culture. Values are expressed as the mean.

	Treatment[1]							*P*-value[2]	
Genus	BrE	BrEPN	BENH	BEPN	BEPBrA	BENL	SEM	T	P
Lactate-utilizing bacteria									
Selenomonas	2.58	2.41	2.51	1.71	2.43	1.89	0.15	0.97	0.11
Megasphaera	1.26	0.39	0.72	0.99	0.35	0.43	0.15	0.45	0.26

[1] A = Brewer's YE > 60% CP with high protein DH

B = Blend of Brewer's YE and Baker's yeast peptone > 65% CP with high and low levels protein and nucleotide DH

C = Blend of Baker's YE > 50% CP with high and medium levels of protein and nucleotide DH

D = Blend of Baker's YE and peptone > 65% CP with high and low levels of protein and nucleotide DH

E = Blend of Baker's YE and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein DH

F = Blend of Baker's YE > 60% CP with medium and low levels of protein and nucleotide DH

[2] *P*-values are reported to show the main effects of treatment (T) and period (P)

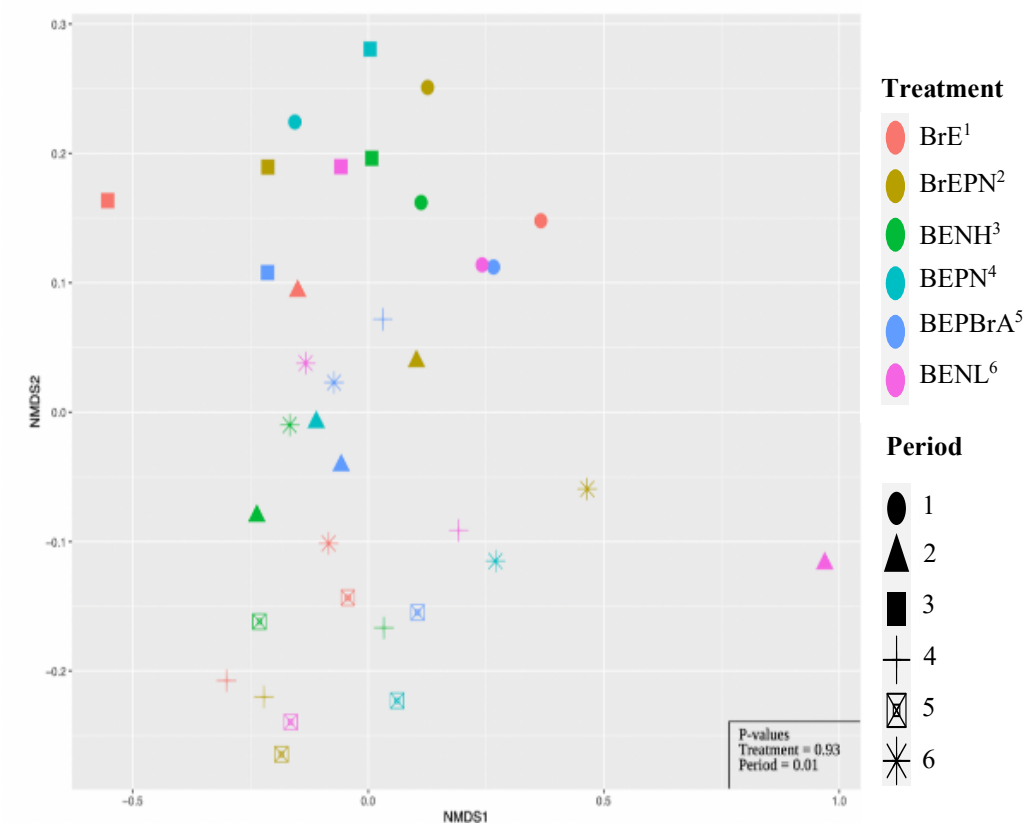

Figure 2.1. Non-metric multidimensional scaling analysis using Bray-Curtis dissimilarity to observe the distance of rumen bacteria in continuous culture when supplemented with six yeast extract treatments: 1) Brewer's yeast extract > 60% CP with a high degree of protein hydrolysis 2) blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels protein hydrolysis and nucleotides 3) blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides 4) blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides 5) blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis 6) blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides, as well as the distance observed between experimental periods.

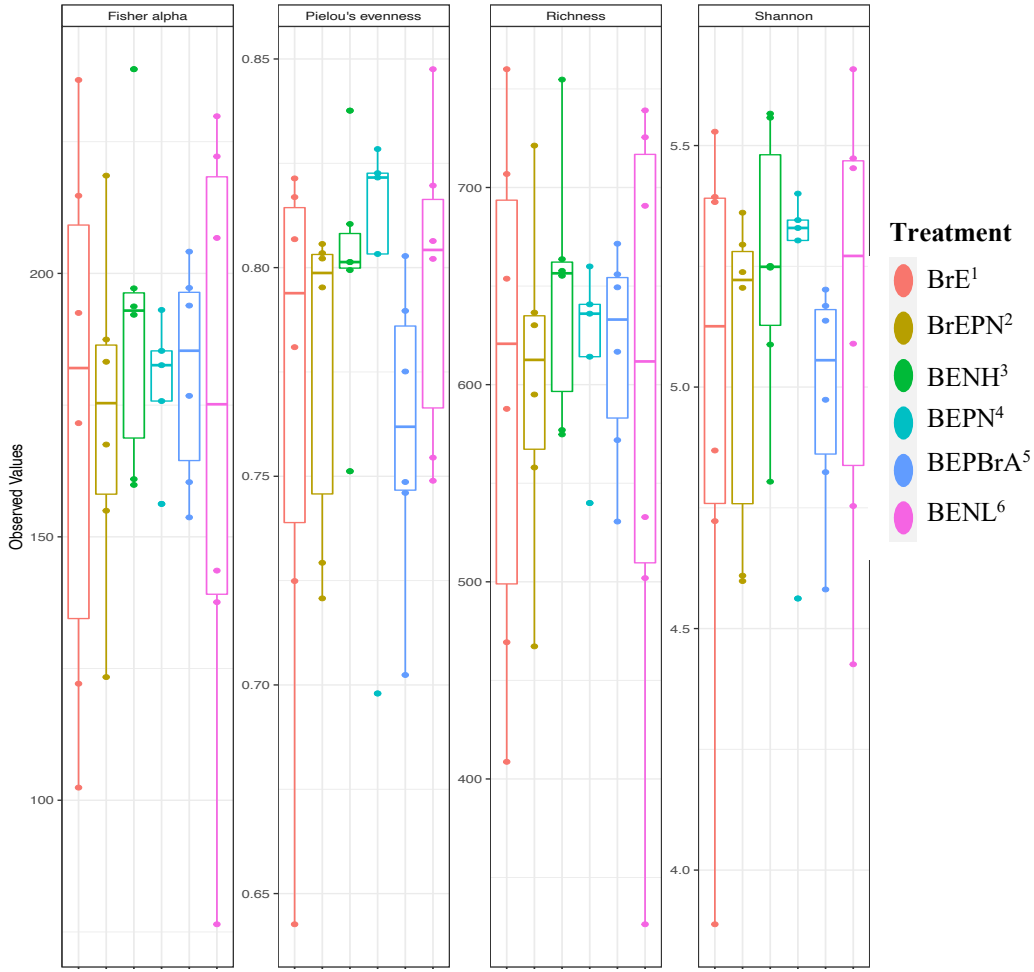

Figure 2.2. Alpha diversity indices (Fisher's alpha, Pielou's evenness, Chao1 richness, Shannon's diversity) of rumen bacteria sampled from the continuous culture fermenters when supplemented with six yeast extract treatments: 1) Brewer's yeast extract > 60% CP with high degree of protein hydrolysis 2) blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with high and low levels protein hydrolysis and nucleotides 3) blend of Baker's yeast extract > 50% CP with high and medium levels of protein hydrolysis and nucleotides 4) blend of Baker's yeast extract and peptone > 65% CP with high and low levels of protein hydrolysis and nucleotides 5) blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis 6) blend of Baker's yeast extract > 60% CP with medium and low levels of protein hydrolysis and nucleotides.

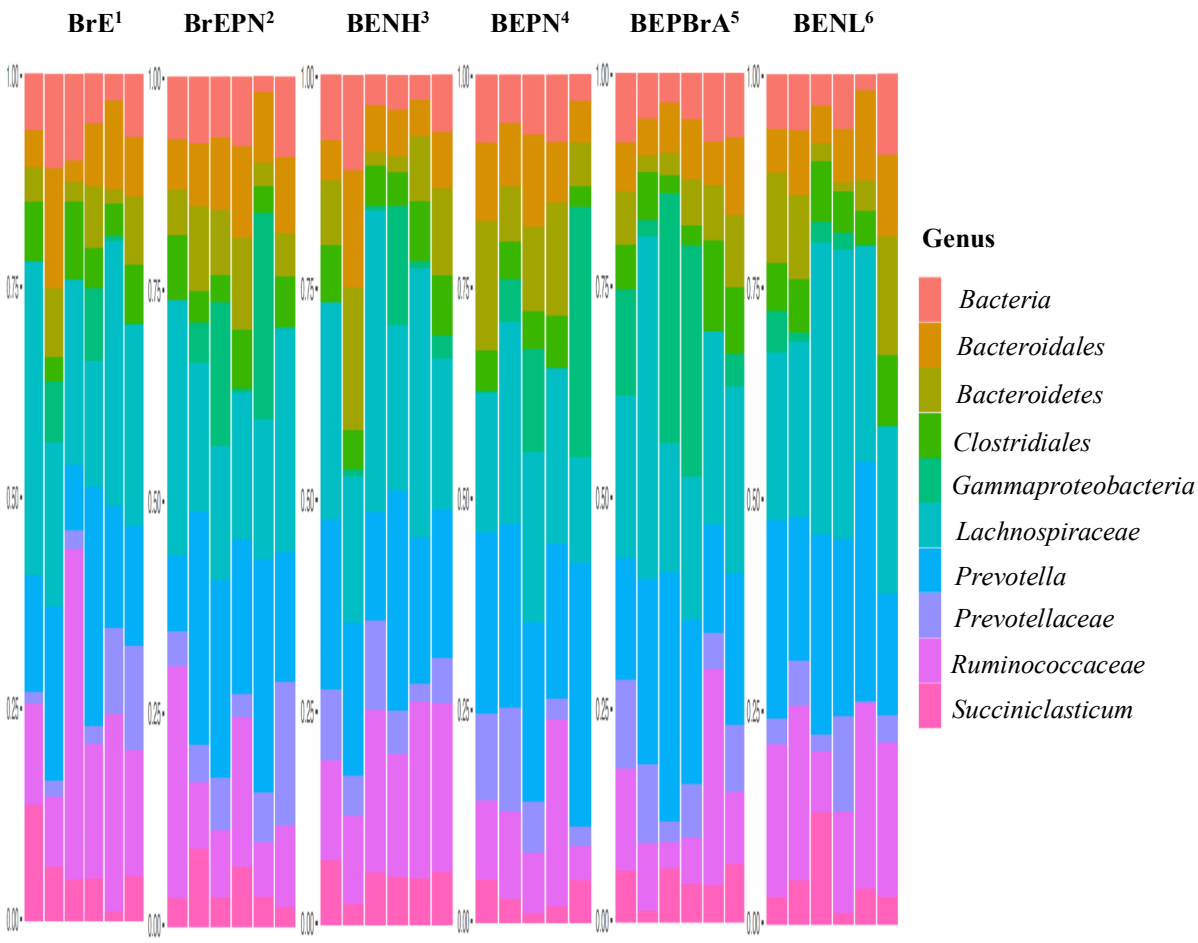

Figure 2.3. Relative abundance at the genus level of the bacterial composition (relative %) when supplemented with six yeast extract treatments: 1) Brewer's yeast extract > 60% CP with a high degree of protein hydrolysis 2) blend of Brewer's yeast extract and Baker's yeast peptone > 65% CP with a mixture of high and low levels protein hydrolysis and nucleotides 3) blend of Baker's yeast extract > 50% CP with a mixture of high and medium levels of protein hydrolysis and nucleotides 4) blend of Baker's yeast extract and peptone > 65% CP with a mixture of high and low levels of protein hydrolysis and nucleotides 5) blend of Baker's yeast extract and peptone and Brewer's yeast autolysate > 50% CP with medium levels of protein hydrolysis 6) blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides. Collapsed by treatment grouped by individual experimental periods.

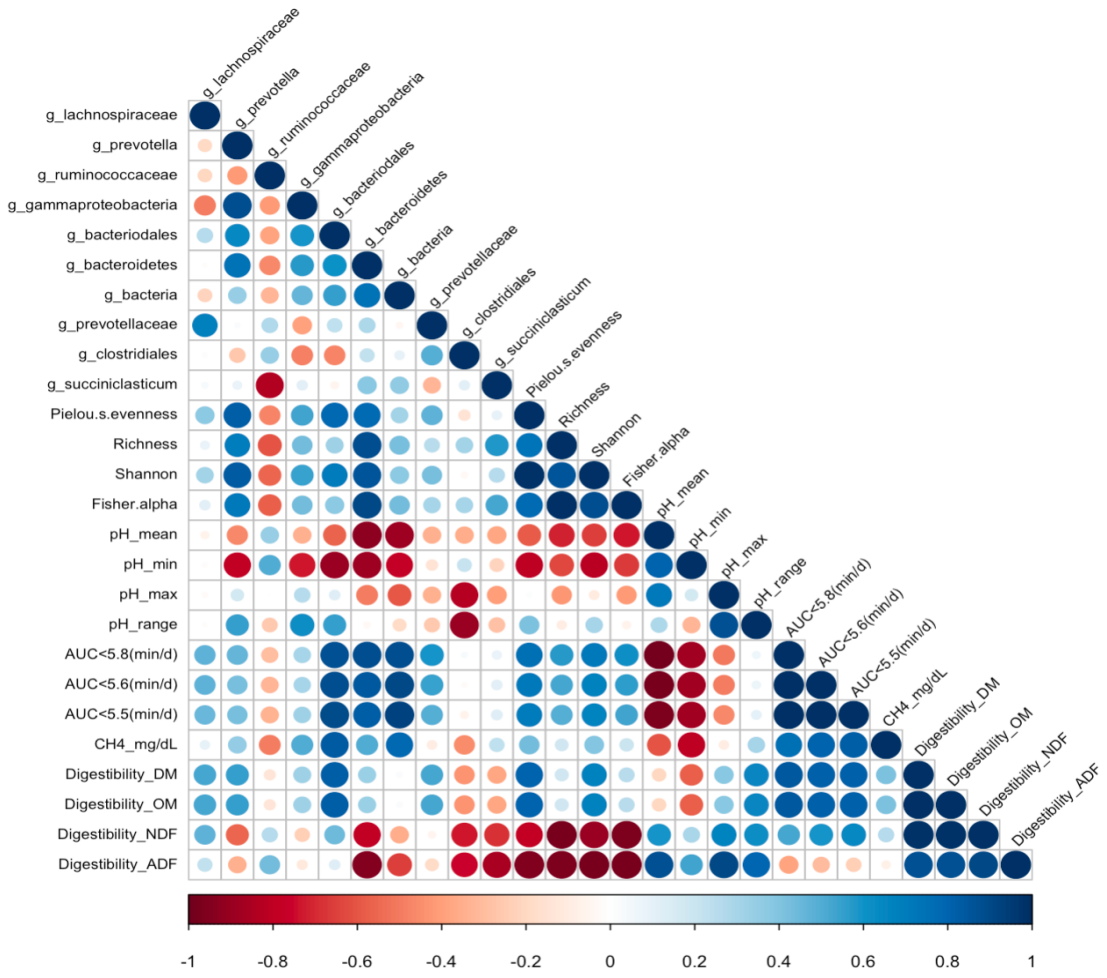

Figure 2.4. Spearman correlation matrix of treatment BrE (Brewer's yeast extract > 60% CP with a high degree of protein hydrolysis) comparing the top ten most abundant bacterial genera (relative %), alpha diversity indices of rumen bacteria, pH measurements of fermentation including area under the curve, methane concentration, and digestibility parameters**.** A positive correlation (closer to 1) is shown by a darker shade of blue and a negative correlation (closer to -1) is shown by a darker shade of red ($P < 0.05$).

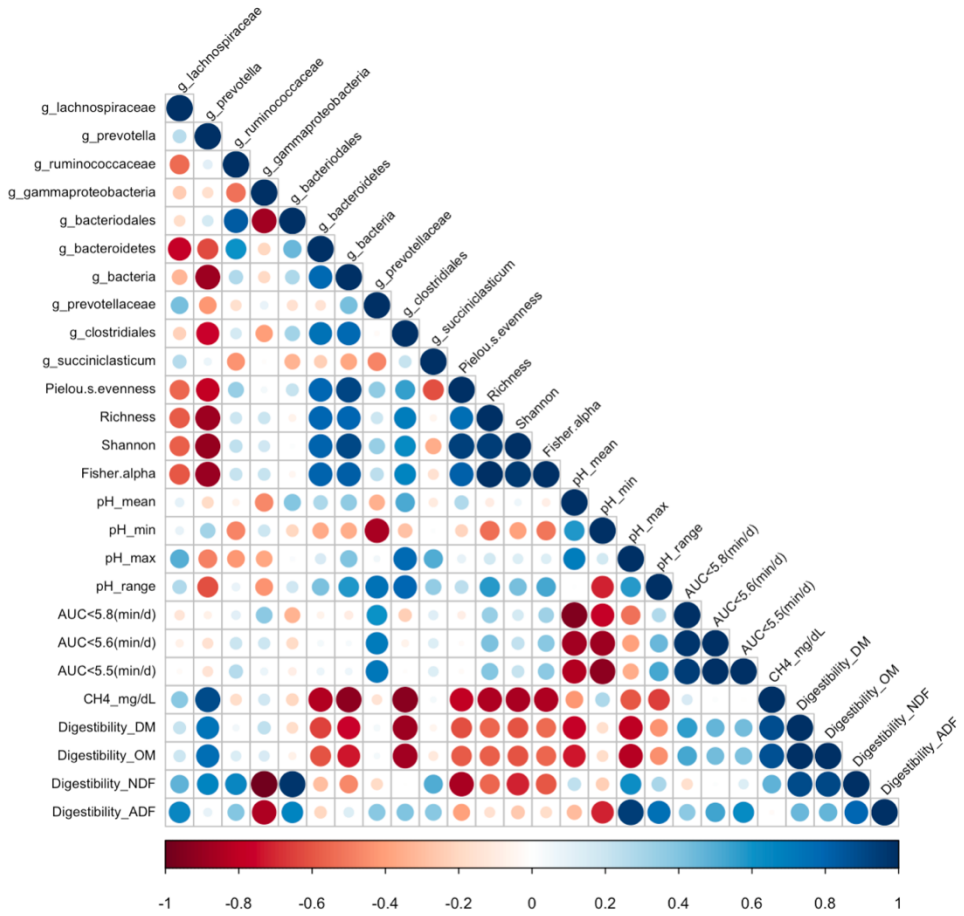

Figure 2.5. Spearman correlation matrix of treatment BENL (blend of Baker's yeast extract > 60% CP with a mixture of medium and low levels of protein hydrolysis and nucleotides) comparing the top ten most abundant bacterial genera (relative %), alpha diversity indices of rumen bacteria, pH measurements of fermentation including area under the curve, methane concentration, and digestibility parameters**.** A positive correlation (closer to 1) is shown by a darker shade of blue and a negative correlation (closer to -1) is shown by a darker shade of red ($P < 0.05$).

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Chapter 3: General Discussion and Conclusions

3.1 General Discussion

The primary goal of this research was to evaluate the effects of six different yeast extracts on the rumen microbiota and rumen environment using *in vitro* methods. This study, which utilized continuous culture fermenters, provided the opportunity to evaluate potential differences among yeast-derived extracts that were different in by-product origin (*i.e*., Baker's yeast or Brewer's yeast), processing (*i.e.,* yeast extract, peptone or autolysate), and degree of hydrolyzation (of both protein and nucleotides). We observed no differences in protozoa and bacteria counts, the rumen bacteria profile or fermentation parameters in response to dietary yeast extract supplementation. However, it is important to note some successes and limitations highlight some areas that warrant further exploration.

3.2 Limitations

Continuous culture fermenters are an established method to simulate rumen parameters and microbial cultures *in vitro*. This method, however, is not without limitations. A major challenge during this study was the inoculation of the fermenters with rumen fluid from fistulated cows. The rumen environment is regulated at body temperature, it is dark, and is mostly free from oxygen. The transfer of live rumen microorganisms from the rumen environment poses a challenging hurdle to overcome as they are exposed to oxygen, light, and external temperatures that are detrimental to their survival. Although methods were taken to circumvent these obstacles (*e.g*., rapid collection times, heated

collection buckets) not every inoculation was successful. Rumen fluid was collected for the experiment from June to February, and was less likely to stabilize following inoculation during the winter months where external temperatures were lower. However, one benefit of utilizing continuous culture is the ability to recollect rumen fluid and re-inoculate the fermenters for a better outcome if the previous inoculation was unsuccessful. The establishment and survival of the rumen microbiota within the fermenters following inoculation were crucial to the success of each experimental period, especially during the first 48 hours. Past studies have minimized the negative impact of external parameters on microbiota during the collection and inoculation phases by utilizing temperature regulated mechanisms (Karnati et al., 2009; Dillard et al., 2018), infusion of N_2 or CO_2 to increase the anerobic conditions (Benedeti et al., 2015; Paula et al., 2017), and minimizing the time spent at and between the collection period and inoculation (Gregorini et al., 2010; Wenner et al., 2017; Dillard et al., 2018). We incorporated these temperature regulated mechanisms and minimized the time spent during and between collections into our experimental design to maximize successful continuous culture operations. As with most continuous culture studies evaluating rumen dynamics, an adaptation period was also included to allow for microbial adjustment within the fermentation vessels. Each experimental period included 7 days for adaptation of the rumen microbiota, allowing for adjustment to diet, treatment, and environment before samples were taken during the sampling days (days 8 – 10 of each period).

Another limitation of continuous culture studies, including our own, has been the ability to maintain protozoal populations within the fermenters. It has been well documented that protozoal populations are difficult to retain in continuous culture systems

(Slyter and Putnam, 1967; Stern and Hoover, 1979; Teather and Sauer, 1988; Karnati et al., 2009) after rumen content collection from fistulated animals. Dual-flow continuous culture fermenters provide a greater ability to evaluate rumen dynamics by controlling liquid and solid passage rates and evaluating nutrient flows throughout the course of the experiment. To facilitate digesta flow from the systems, most fermenters have established overflow spouts. It has been observed that the use of overflow spouts very quickly lose protozoa from the system (Slyter and Putnam, 1967; Abe and Kumeno, 1973; Stern and Hoover, 1979). This is due to the lag time of protozoal generation being slower than that of the fermenter system's turnover time (Teather and Sauer, 1988). To maintain protozoa within the fermenters, studies have developed filters for use in dual-flow continuous culture (Hoover et al., 1976; Teather and Sauer, 1988; Karnati et al., 2009). The physical application of this filtration device was difficult for use with rumen simulation as feed particles would clog the filter (Teather and Sauer, 1988; Karnati et al., 2009) and the need for filter replacement during the fermentation periods were necessary (Karnati et al., 2009). Although the filters did facilitate an improved retention of protozoa counts in the fermenters, replacement of these filters throughout the fermentation period is not ideal as the opening of the system allows aeration to an anerobic culture that contains cellulolytic species highly intolerable of oxygen (Lynd et al., 2002). Further adaptation of the filters by the Greenwood and Kraft labs as well as the inclusion of two filters within each system eliminated the need for filter replacement during the experiments and kept the fermenters as a closed system for the entire 10-day period. However, even this updated design is susceptible to blockage, and the flow rates between each individual filter and between fermenters were not equal and frequent priming of the filtration tubing was necessary

during the first few days of each period to maintain a steady flow within each fermenter. This is still largely due to the clogging of the filters with excess feed particles and the viscosity of the fluid immediately after inoculation. Our modified filters achieved consistent flow rates after the first 48 hours as well as retained protozoa counts in the fermenters. This achievement was accomplished by maintaining rumen fluid levels just below the overflow spout and preventing excessive overflow from the spout, limiting protozoa washout. However, this is perhaps the reason that DM values of overflow effluent were low compared to other studies (Benedeti et al., 2015; Salfer et al., 2018) but were similar to Karnati et al. (2009) who utilized filtration within their systems.

Our fermenter vessels were much larger in size compared to many other continuous culture studies. Larger fermentation vessels facilitated the ability to use a larger feed grind size (2 mm) as well as feed a fresh (non-dried) ration to the fermenters as opposed to dried or pelleted feed, which is more representative of *in vivo* practices. The ration provided to the fermenters was the same as that fed to the fistulated cows from which we collected rumen fluid. Our study fed 109 g of DM/d of ration to each fermenter split into two separate feedings which was within the accepted published range (Karnati et al., 2009; Wenner et al., 2017; Moya et al., 2018; Miller-Webster et al., 2002). One study suggested utilizing 75g of DM/L/d of fermenter volume, however this was not a study observing the supplemental effects of yeast (Salfer et al., 2018), and this recommendation is likely also dependent on the base diet profile.

3.3 Future Research

Provided with unlimited time and budget, the ability to further investigate the impact of the differences among the yeast extract treatments within our study would be possible. One example would be to include an additional fermenter to enable the inclusion of a control treatment, where no yeast is added. The ability to include a no yeast control in a future study would not only allow the comparison between each of these yeast extracts but also would provide how these yeast extracts alter rumen function beyond the base diet. In the current study, none of the treatments resulted in a response compared to the others; however, it is plausible that had the yeast extract treatments been compared against a control, differences would have been observed.

Continuous culture is a method which allows researchers to conduct studies evaluating different diets and supplements without causing any adverse effects to the live animal. Although there are many benefits in using this method, the ability to monitor and sample for animal health and production parameters is impossible. To further evaluate the impact of these dietary yeast extract treatments on health and production in dairy cows, it would be advisable to conduct an *in vivo* study utilizing lactating cows to observe changes in animal health parameters (*e.g.*, via lipopolysaccharide (LPS) and serum amyloid A levels), and production parameters (*e.g.*, DM intake, milk yield, and milk component %). Many studies have examined inflammation-associated parameters as indicators of digestive imbalance. However, only a few of these studies have assessed these parameters when evaluating yeast or yeast components such as manno-oligosaccharides (Diaz et al., 2018; Silberberg et al., 2013). Some research does support the ability of yeast to alleviate the decline in ruminal pH which can cause subacute ruminal acidosis (SARA). Evidence

suggests that SARA increases the lysis of gram-negative bacteria releasing free LPS which stimulate an immunogenic response in the animal (Gozho et al., 2007). Inflammation stimulated by increased LPS concentrations increase acute phase proteins such as serum amyloid A (Gozho et al., 2007). Conducting an *in vivo* study where SARA is induced within lactating dairy cows would provide the opportunity to collect blood samples for analysis of these inflammation markers (*i.e*., LPS and serum amyloid A) and would provide the opportunity to compare the differences among the yeast extract treatments and their influence on the alleviation of inflammation.

Additionally, dietary yeast can promote the growth of cellulolytic bacteria and lactate- utilizing bacteria, both of which can impact production performance in lactating dairy cows. Future studies may focus on DM intake, milk yield, and milk components. One study observed an increase in milk yield when dairy cows were supplemented with yeast compared to the control but did not observe any differences in milk components (Faccio-Demarco et al., 2019). This aligns with the findings from a meta-analysis examining the impact of dietary yeast on rumen dynamics (Desnoyers et al; 2009). However, not all studies resulted in the same conclusions. A study conducted by Kalmus et al. (2009) that investigated the production response of lactating dairy cows supplemented with dietary yeast observed differences in milk fat and milk protein components but did not see any change in milk yield. The variability in the literature has been attributed to yeast strain and dosage in each experiment (Vohra et al., 2016) and indicates that the composition of the yeast is an important factor to consider when supplementing yeast to dairy cows. For this reason, it would be advantageous to conduct a study that compares the differences of origin, processing, and degree of hydrolyzation of the yeast extracts in the current study.

3.4 Conclusions

The thesis presented herein outlines the impact of different yeast extracts on simulated rumen function and microbiota using *in vitro* continuous culture fermenters. This research contributed to the growing body of knowledge examining feeding yeast to ruminants by evaluating protozoa and bacteria counts, bacterial composition and diversity, and the impact on rumen fermentation parameters such as pH, methane, and digestibility. This study included treatments containing either a combination of Baker's and Brewer's yeast processed as extracts, peptones or autolysates with differing protein and nucleotide hydrolysis. There were no differences on the rumen microbiota or fermentation parameters when supplemented with any of the six different yeast extracts, which indicates that the yeast extract treatment which is the most economical to produce would perform similarly to the yeast extract treatment that is the least economical. Future research should compare these yeast extracts with a control to establish its effectiveness relative to a diet that excludes yeast, as well as a dose response study to measure efficacy thresholds. Moreover, conducting an *in vivo* experiment would provide a more detailed understanding of the impact that the yeast extracts may have on animal health and production.

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