# THE EFFECT OF SUPPLEMENTING NATIVE RUMEN MICROBES ON MILK PRODUCTION OF DAIRY CATTLE

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## ABSTRACT

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Dairy cows are commonly fed direct-fed microbials (DFM) to improve milk production and efficiency. Most DFM are not native to the dairy cow rumen which may limit their ability to interact with the native microbiome. We evaluated the effects of two DFM supplements containing 4 native rumen microorganisms on the production of dairy cows. Ninety Holstein cows (45 ± 10 kg milk/d, mean ± standard deviation; 40% primiparous) were fed a common diet. After 14 d, they were blocked by parity, days in milk, and energy corrected milk (ECM) per unit of metabolic body weight. Within block, cows were randomly assigned to treatments, which were top-dressed daily for the next 112 d. Treatments were 150 g of ground corn mixed with 1) no live DFM (CON), 2) 5 g of a live DFM (Galaxis 2.0; G2), and 3) 5 g of a live DFM (Galaxis 2.0 Plus; G2P). G2 and G2P were products of Native Microbials Inc. (San Diego, CA) and contained the same organisms but in different concentrations. Supplementation with DFM did not alter yield of total milk, protein, or fat, but slightly decreased body weight gain and body condition score gain with no difference between G2 and G2P. DFM tended to decrease dry matter intake (DMI) but did not significantly improve feed efficiency (ECM/DMI). DFM did not alter digestibility of fiber, starch, protein, or fat, and did not alter concentrations of glucose or non-esterified fatty acids but tended to decrease concentration of insulin in plasma. DFM decreased somatic cell counts in milk with no difference between G2 and G2P. In conclusion, supplementation with native DFM had little impact on milk production and efficiency.

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## KEY TO ABBREVIATIONS

AA = Amino acid

ADF = Acid detergent fiber

ADG = Average daily gain

ATP = Adenosine triphosphate

ATTD = Apparent total tract digestibility

BCS = Body condition score

BHBA = Beta-hydroxybutyrate

BW = Body weight

CAPE = Captured energy

CFU = Colony forming units

CON = Control treatment

CP = Crude protein

DFM – Direct-fed microbial

DIM = Days in milk

DMI = Dry matter intake

ECM = Energy corrected milk

FEEDE = Feed energy

G2 = Galaxis 2.0 treatment

G2P = Galaxis 2.0 Plus treatment

GIT = Gastrointestinal tract

iNDF = Indigestible neutral detergent fiber

IOFC = Income over feed cost

LAB = Lactic acid producing bacteria

LUB = Lactic acid utilizing bacteria

MCP = Microbial protein

MILKE = Milk energy

MOS = Mannan-oligosaccharides

MUN = Milk urea nitrogen

NDF = Neutral detergent fiber

NEFA = Non-esterified fatty acid

OM = Organic matter

SARA = Subacute ruminal acidosis

SCC = Somatic cell count

SD = Standard deviation

SEM = Standard error of the mean

TMR = Total mixed ration

VFA = Volatile Fatty Acid

# CHAPTER 1

# INTRODUCTION

The rumen microbiome is a robust community of microorganisms that plays an integral role in many functions of the ruminant. These microorganisms convert feed to usable energy, producing approximately 70% of the energy supplied to the animal, as well important precursors for substrates essential to milk production and composition (Bergman, 1990). Improving the function of the rumen microbiome by supplementing additional, beneficial organisms could improve its energy and substrate production. For these reasons, DFM are commonly used in the dairy industry to manipulate and improve the function of the rumen microbiome and therefore, overall milk production and efficiency.

Many studies have evaluated the effect of DFM supplementation in ruminants (Yoon and Stern, 1995; Chaucheyras-Durand et al., 2008; Seo et al., 2010). Many modes of action have been proposed for DFM and include moderating pH, moderating redox potential, and improving nutrient digestibility (Chaucheyras-Durand et al., 2008; Yoon and Stern, 1995). DFM sometimes increase milk production (Boyd et al., 2011; Nocek et al., 2003) and lower rumen pH (Nocek et al., 2002), but results are inconsistent and vary by DFM species, strain, dosage, frequency, and animal physiological status (Chaucheyras-Durand et al., 2008).

Most organisms in commercial DFM are not native to the rumen (Henderson et al., 2015). The strong resilience of the rumen microbiome may limit interaction with these non-native organisms (Weimer, 2015). For this reason, supplementing native rumen microorganisms may improve DFM effects. Research evaluating native rumen microorganism supplementation and its effects on production or efficiency of ruminants is limited. A study by Goetz et al. (2021) supplemented a DFM with two native rumen organisms to dairy cows and reported no significant effects. When completing a retrospective analysis, Goetz et al. (2021) found low and high

producing cows responded differently to native DFM. Therefore, we were interested in how DFM containing native microorganisms affect milk production and nutrient digestibility in dairy cattle.

# CHAPTER 2

# LITERATURE REVIEW

## The Rumen Microbiome

The rumen microbiome is the community of prokaryotes (bacteria and archaea) and eukaryotes (fungi and protists) that exist in a symbiotic relationship with the ruminant. One mL of rumen fluid contains approximately 25 billion bacteria and archea, 10 million protozoa, and 10 thousand fungi (Leedle et al., 1982; Clarke et al., 1965; Joblin, 1981). When considering the total contents of the rumen, over three quadrillion ($1x10^{15}$) microbes inhabit the environment (Jurgens, 2002). For many years, species identification was difficult as most rumen microorganisms are unculturable. With recent advances in genome analytics, researchers have identified over 5,000 unique microorganisms (Stewart et al., 2019).

The rumen microbiome provides many advantages to the ruminant. These microorganisms enable the cow to ferment cellulosic material, which would be otherwise undigestible, and form volatile fatty acids (VFA). Microbes ferment other organic matter as well. Fermentation accounts for approximately 70% of the energy supplied to the animal (Bergman, 1990). VFA also serve as important precursors for substrates essential to milk synthesis. Rumen microbes also produce vitamins and amino acids through fermentation (Moran, 2005). Researchers and the industry are interested in understanding the roles, interactions, limitations, and potential for manipulation of the rumen microbiome.

### *Rumen Microbiome Establishment*

The species that form the rumen microbiome community are established early in dairy cattle. Within hours of birth, bacteria and yeast are already colonizing the rumen (Rey et al., 2014; Abecia et al., 2014; Guzman et al., 2015). These microorganisms are introduced to the calf through their dam, other adult animals, and the surrounding environment (Ziolecki and

Briggs, 1961). Following initial colonization, the species and concentrations of the rumen community shift depending on diet, management, and unknown innate animal factors (Rey et al., 2014; Bryant and Small, 1960; Eadie, 1962; Malmuthuge et al., 2014). Within 3-4 weeks after birth, the microbial community reaches a state of stabilization where many of the present microorganisms form the core successional microbiome (Rey et al., 2014; Abecia et al., 2014; Guzman et al., 2015; Furman et al., 2020). The rumen core successional microbiome consists of species that maintain a high persistency throughout life. Although it may experience slight composition changes depending on age and diet, it is highly resistant to perturbations (Furman et al., 2020; Moraïs and Mizrahi., 2019). Microorganisms that are early rumen colonizers (0 – 10 days after birth) are more likely to persist and be a part of the core successional microbiome (Furman et al., 2020). The species that compose the core successional microbiome may affect animal health, production, and efficiency throughout their life. Recent research suggest genetics may also be important in rumen colonization (Wallace et al., 2019).

***Rumen Microbiome Grouping***

With such a vast number of microorganisms, it can be difficult to describe their roles and interactions. Moraïs and Mizrahi (2019) propose evaluating the rumen microbiome using functional grouping and community states. A functional group can consist of many different strains, species, or even kingdoms of microorganisms that serve the same function in the rumen. For example, all microbial species that utilize pyruvate to produce propionate compose one functional group. Together, functional groups form the community state. Community states are the stable microbiome composition that may alter slightly but primarily resists change unless drastically perturbed. Community states may have different compositions between animals but still carry out the same processes. Henderson et al. (2015) suggests that microbial interactions

within community states do not follow exact relationships, but instead demonstrate flexible associations between members of functional groups. This flexibility aids the ruminant in adapting to and utilizing various diets (Henderson et al., 2015). Functional and community groups help to simplify comparison of rumen microbial compositions that although different, still complete the same tasks.

***Energy Production by the Rumen Microbiome***

The primary function of the rumen microbiome is fermentative digestion of feed to harvest energy. Through fermentation, microorganisms also produce waste products including VFA (France and Dijkstra, 2005). VFA serve as a crucial source of energy for the ruminant. The rumen microbiome produces approximately 70% of the energy needed by the host, mainly through VFA production (Bergman, 1990).

Once produced, VFA are absorbed through the rumen epithelium into the bloodstream and transported to tissues (Stevens, 1970; Dijkstra et al., 1993). Absorbed propionate is primarily used in the liver for gluconeogenesis, providing almost all the glucose for the ruminant. Acetate and butyrate undergo oxidation via the citric acid cycle in tissues for energy utilization (France and Dijkstra, 2005). Additionally, acetate serves as a primary substrate for lipogenesis. The pattern of VFA produced in the rumen is directly linked to the rumen microbiome composition (Dijkstra et al., 1994).

The rumen microbiome also serves as a valuable source of protein to the animal. Rumen microbes break down ingested protein to amino acids and ammonia to form microbial protein (MCP). Rumen microbes regularly leave the rumen via attachment to feed particles or the ruminal fluid (Moran, 2005). In the small intestine, MCP is broken down to AA and absorbed for use by the animal, accounting for approximately two thirds of the ruminant's total absorbable

protein (Tas et al., 1981; Storm et al., 1983). Absorbed amino acids are partitioned to tissues for use including milk protein synthesis, growth, and maintenance (Henry and Morrison, 1928). Overall, the rumen microbiome plays a critical role in energy and protein status of the dairy cow.

***Dietary Effects on Microbiome Composition***

Diet is integral to the diversity and richness of the rumen microbial community. Although the rumen core microbiome remains stable through perturbations, the concentrations of member species maintain some plasticity (Belanche et al., 2019). When diets shift, plasticity allows the microbiome to adjust according to the nutrient substrates available. For example, dairy cows consuming fiber rich diets had increased concentrations of cellulolytic protozoa, fungi, and methanogens when compared to consuming starch rich diets (Belanche et al., 2012). Compared to eating a pasture diet, sheep consuming a starch rich diet had a less diverse and dense rumen microbiome but produced more VFA. When transitioned to a pasture diet, the sheep's microbiome adjusted to the newly available nutrients and was more diverse and denser (Belanche, 2019). Diet contributes greatly to the composition, concentration, and complexity of the rumen microbiome.

***Microbiome and Rumen pH***

The microbiome is an important regulator of rumen pH. Maintaining the typical pH of the rumen (5.6-7) is critical for the survival and efficacy of the rumen microorganisms (Grünberg and Constable, 2009). When VFA accumulate in the rumen faster than they can be absorbed by the rumen epithelium, typically from ingesting large quantities of highly fermentable carbohydrates, rumen pH begins to drop (Britton and Stock, 1991). When pH falls between 5.2-5.6 for an extended period, cows experience subacute rumen acidosis (SARA). SARA decreases microbial community richness, diversity, fibrolytic activity, buffering capacity, and rumen

motility (Stewart, 1977; Lorenz, 2016; McCann et al., 2016). Low rumen pH stimulates the growth of lactic acid producing bacteria, generating lactate. Lactate has a lower pKa than VFA (pKa 3.9 vs. 4.9) making it more difficult for the rumen epithelium to absorb (Nagaraja and Titgemeyer, 2006). If pH is not stabilized at this point, a spiraling effect may occur. When pH drops below 5.2, described as acute acidosis, inflammation, erosion, and ulceration of the ruminal epithelium can occur (Mutsvangwa et al., 2002; Lorenz, 2016). Understanding the relationship between diet, rumen microorganisms, and rumen pH is important to maintain healthy conditions for animal and microbial success.

***Rumen Microbiome Manipulation***

Across cows, the core microbiome may be similar, but each is unique in its concentration and composition of species. Most microbes composing the rumen core microbiome are early colonizers, almost all introduced during the days following birth (Furman et al., 2020). This suggests that early intervention can have lasting effects on the core rumen microbiome. Research has shown that manipulating feed management in early life formed unique microbiomes that persisted later in life (Yáñez-Ruiz et al., 2015; Abecia et al., 2014). Furman et al. (2020) demonstrated that different birth delivery methods (cesarean section compared to vaginal) affected rumen microbial composition that endured through life. Research is still needed to determine best timeframes, methods, and microorganisms for effective and persistent intervention in calves.

Once established, the core microbiome is difficult to significantly alter. In a study by Weimer et al. (2010), 2 pairs of cows whose bacterial community composition were significantly different underwent exchange of 95% of their rumen contents. These cows reestablished their pre-exchange pH and VFA profiles within 24 hours and bacterial community composition within

14-61 days. Zhou et al. (2018) conducted complete rumen content exchange between 8 pairs of beef steers of varying feed efficiencies. Like Weimer et al. (2010), Zhou et al. (2018) found the animal's rumen microbiome reestablished to similar compositions as prior to exchange. This research suggests that mature ruminants maintain a strong specificity for their core microbiome and even drastic perturbations cannot completely alter its composition. This strong specificity of the rumen microbiome must be considered when considering DFM supplementation.

## Direct-Fed Microbials

Direct-fed microbials **(DFM)** have been researched and used in the dairy industry at length. DFM have been defined as products containing viable bacteria and/or yeast by the United States Food and Drug Administration (1995). DFM were originally conceived as a route to improve performance and health by manipulating the post-ruminal microbiome (Fuller, 1989). As the rumen microbiome's role in energy production and overall health was better understood, DFM effects on rumen function became a major interest to researchers and farmers.

DFM products have been researched to evaluate their modes of action, rumen effects, and animal production responses. These studies have generated inconsistent results. Additionally, the ability to compare studies is difficult due to many confounding variables influencing the effect of DFM. Many studies use different microorganisms, dosage, frequency, delivery form, diets, and animals of various physiological states. These factors can all affect the rumen microbiome and therefore, the effectiveness of DFM (Ban and Guan, 2021). This makes drawing clear conclusions difficult and therefore when evaluating the literature, readers should be aware of these factors. Regardless, results from DFM supplementation studies suggest potential to benefit rumen health, performance, and efficiency.

***Yeast and the Rumen***

Yeast is commonly included in DFM because of their role as "oxygen scavengers". Yeasts are facultative anaerobes, surviving in environments with or without oxygen present. The rumen is a primarily anaerobic environment, but dissolved oxygen can be introduced to the rumen fluid through eating, drinking, salivating, or rumination (Newbold et al., 1996). Dissolved oxygen can severally inhibit the growth and performance of anaerobic ruminal bacteria (Newbold et al., 1996). Yeast can scavenge the rumen for oxygen and utilize it for respiration. By eliminating dissolved oxygen, yeast promotes the anerobic rumen environment and subsequently, the success of bacteria, particularly fibrolytic species (Chaucheyras-Durand et al., 2008). A study by Newbold et al. (1996) exhibited this relationship in fermentation simulators by supplementing mutants of the yeast species *S. cerevisiae* that were unable to metabolize oxygen. Mutant *S. cerevisiae* were unable to promote bacterial populations or $O_2$ uptake whereas strains with normal oxygen consuming ability increased bacterial abundance and $O_2$ uptake. As a facultative anaerobe, yeasts can play a unique role in promoting rumen microbial success.

Yeast's uptake of oxygen is not only important for preserving anerobic conditions, but also maintaining the rumen redox potential. Rumen bacteria, particularly fibrolytic species, require low redox potential to complete metabolic functions of fermentation (Huang et al., 2018). Julien et al. (2020) demonstrated that anerobic ruminal bacteria diversity and richness is closely related to the rumen redox potential. Redox potential in the rumen is directly linked to, and increases, with the presence of dissolved oxygen. Therefore, yeast's consumption of dissolved oxygen may help to maintain low redox potential and promote bacterial growth. Live yeast also produce growth factors such as vitamins and organic acids that are utilized by the bacteria,

further decreasing redox potential (Jouany, 2006; Chaucheyras-Durand et al., 2008). In vitro studies have demonstrated that feeding live yeast decreases the redox potential in the rumen of lambs (Chaucheyras-Durand and Fonty, 2002; Mathieu et al., 1996) and dairy cows (Křížová et al., 2011). This in vitro research suggests DFMs containing yeast can be used to maintain a low rumen redox potential and effectively promote microbial performance.

Yeast may also stabilize rumen pH by promoting viable bacterial growth. Yeast produce growth factors such as amino acids and peptides that promote growth of rumen lactate-utilizing bacteria (LUB; Chaucheyras-Durand et al., 2008). LUB can ferment lactic acid, a major contributor to acute acidosis, and increase rumen pH. Additionally, yeast can stimulate protozoa that utilize lactate, compete with amylolytic bacteria, and ferment starch at a slower rate (Brassard et. al., 2006; Mendoza et al., 1993; Williams and Coleman, 1997). Studies have reported that feeding supplemental live yeast increased average, minimum, and maximum rumen pH (Křížová et al., 2011; Bach et al., 2007; Nocek et al., 2003; Thrune et al., 2009) whereas others (Yoon and Stern, 1996; Nocek et al., 2002) saw no difference. Bach et al. (2007) reported that cows supplemented with live yeast had shorter bouts of rumen pH below both 5.6 and 6.0. Stabilizing rumen pH and stimulating bacterial growth can promote optimum animal health, digestion, and performance. Overall, yeast may improve rumen conditions by maintaining an adequate anaerobic environment, low redox potential, and moderating pH.

***Species of Yeast as DFM***

The most studied supplemental yeast species for dairy cattle is *Saccharomyces cerevisiae*. *S. cerevisiae* is a common yeast used in industrial processes from baking to beer making and is not native to the rumen. Supplementation of live *S. cerevisiae* to cattle has garnered inconsistent results (Yoon and Stern, 1996; Doreau and Jouany, 1998; Tesfaye and Hailu, 2019).

Supplemental *S. cerevisiae* has increased milk yield in goats (Stella et al., 2007) and decreased milk yield for dairy cows (Rossow et al., 2018). Both studies also reported decreased milk fat concentrations. In comparison, similar studies found no difference in milk yield (Maamouri and Ben Salem, 2021; Hossain et al., 2014) but some reported increased milk protein concentrations (Rossow et al., 2018; Hossain et al., 2014). When evaluating *S. cerevisiae*'s effect on digestion, several researchers found no change in ATTD of NDF, OM or ADF (Yoon and Stern, 1996; Chung et al., 2011; Doreau and Jouany, 1998) but Chung et al. reported lower ATTD of CP (2011). Chung et al. (2011) also detected no effect on animal's BW or BCS change. Research reporting the effects of *S. cerevisiae* on cow efficiency was not found at the time of this literature review.

The inconsistent results of supplementary *S. cerevisiae* may be tied to several factors. Dosing of DFM is influential on its effect. Nocek et al. found that various doses of the same strain of *S. cerevisiae* had differing effects on rumen pH and nutrient digestion (2002). Additionally, many studies feed different strains of *S. cerevisiae*, which may act differently. Chung et al. (2011) reported two strains of supplemental *S. cerevisiae* had different effects on rumen pH and VFA concentration in nonlactating dairy cows. Other contributing factors that have not been explicitly evaluated include stage of lactation, parity, and health status of supplemented animals.

Research into the yeast species *Pichia kudriavzevii* as a DFM is limited. Current research is primarily focused on *P. kudriavzevii*'s role as an aflatoxin detoxifier. When fed TMR contaminated with aflatoxin, dairy cows supplemented with *P. kudriavzevii* had increased DMI, but did not differ in BW gain or digestibility of feed (Intanoo et al., 2020). In chickens, supplemental *P. kudriavzevii* did not affect ADG in the basal diet but did improve ADG of

chickens fed aflatoxin contaminated feed (Magnoli et al., 2017). A study by Geotz et al. (2021) found supplemental *P. kudriavzevii* and *C. beijerinckii* (a bacteria) could have beneficial milk yield responses based on cow's production level. Additional research of supplementary *P. kudriavzevii* for ruminants is needed to better understand its effectiveness as a direct-fed microbial.

Overall, supplementation of yeast to the diet of dairy cattle has inconsistent but promising results. Additional research is needed to best understand the interactions of yeast strains, dosage, and frequency for dairy cattle DFM.

***Bacterial DFM***

Bacterial DFMs have been used extensively in the dairy industry. The bacterial species used in DFM vary greatly but are commonly divided into three groups: lactic acid producing bacteria, lactic acid utilizing bacteria, or other species. It is hypothesized that these bacteria may moderate the rumen environment, microbiome, and energy substrate production. Recent research also suggests bacterial DFM may participate in competitive exclusion and immunomodulation (McAllister et al., 2011). Overall, bacteria appear to be an important consideration in DFM formulation.

***Lactic Acid Producing Bacteria (LAB) as DFM***

Lactic acid producing and utilizing bacteria are commonly included in DFM together due to the symbiotic relationship. Through the fermentation of feedstuffs, LAB produce lactic acid. This lactic acid can then be used by lactic acid utilizing bacteria (LUB) to produce energy substrates such as VFA. Additionally, increasing the concentration of lactic acid in the rumen stimulates LUB growth (Yoon and Stern, 1996). An increased LUB population can moderate lactate concentration, stabilizing the rumen pH and rumen microflora.

In the small intestine, LAB may benefit the ruminant through competitive exclusion with pathogenic organisms. Some pathogenic organisms attach to the epithelial wall of the small intestine, promoting their growth and decreasing peristaltic removal (Jones and Rutter, 1972). LAB can compete for sites of adherence to the intestinal epithelial cells or produce products that limit other organisms' ability to adhere. This decreases the prevalence and success of pathogenic organisms colonizing the small intestine. Strains of *Lactobacillus* have been found to inhibit colonization of epithelium cells by *E. coli* and *Salmonella* strains through competitive adhesion or producing adhesion limiting products (Sherman et al., 2005; Chen et al., 2007). Mature biofilms formed in the GIT by well established organisms can limit bacterial DFM competition. Thus, McAllister et al. (2011) suggests that DFM may have a greater competitive potential in the GIT of young animals who have not yet formed complex biofilms. Overall, through competitive exclusion with pathogenic organisms, LAB can beneficially influence GIT health.

LAB can impact the ruminant's immune system through both a direct antibacterial effect and indirectly enhancing immune responses. All major bacterial groups produce bacteriocins, small peptides and proteins that inhibit competitor bacteria's growth (McAllister et al., 2011). Bacteriocins typically target bacteria similar to the producing strain through varying mechanisms including membrane disruption or nucleic acid degradation (Riley and Wertz, 2002). Due to their prevalence, bacteriocins are hypothesized to play a role in rumen microbial interactions but little research exists investigating this. Bacteriocins produced by strains of *E. faecium* and *S. bovis* have been reported to inhibit some bacterial species' growth (Mantovani et al., 2002). Research by Lee et al. (2002) and Lima et al. (2009) found bacteriocins may be as effective as an ionophore in limiting rumen methane production or AA degradation. Similar to treatment with other antimicrobials, bacteria may become resistant to bacteriocins. This warrants consideration

when formulating and feeding DFM. Additional research of bacteriocins and their role in the ruminant may improve the selection of bacterial species for DFM.

The last believed mode of action of LAB is immunomodulation in the GIT. Research in poultry and cell culturing has indicated DFM may enhance GIT immune response. These indicators include enhanced innate, humoral, or cellular responses of the immune system (Erickson and Hubbard, 2000; Isolauri et al., 2001; Miettinen et al., 1996; Tejada-Simon and Pestka, 1999; Haller et al., 2001). There is little research in ruminants regarding DFM's influence on the immune system, but species, dose, and other factors likely contribute (McAllister et al., 2011). Investigation into the relationship between supplemental bacteria and the ruminant's immune system is an area of needed research.

Lactic acid producing bacteria play several beneficial roles in the ruminant suggesting they may be useful in DFM. Through their symbiotic relationship with LUB, LAB may promote rumen bacterial growth. Additionally, LAB may improve the host's health both directly and indirectly through competitive exclusion, bacteriocins, and immunomodulation. These aspects present reasonable arguments for researching and utilizing LAB in DFM for ruminants.

***Lactic Acid Utilizing Bacteria (LUB) as DFM***

Lactic acid utilizing bacteria are common in the rumen. These species ferment lactic acid and convert it to various substrates, primarily propionate. In ruminants, propionate serves as the major precursor for glucose production in the liver (Huntington, 1989). Therefore, feeding DFM with LUB may be a route to improve energy status of dairy cows. Some studies (Stein et al., 2006; Weiss et al., 2008) saw increased ruminal propionate molar concentrations when supplementing *Propionibacterium* (a LUB) in dairy cattle, whereas Kenney (2013) and Ghorbani et al. (2002) saw no effect in feedlot cattle. DFM dosage likely plays an important role in

propionate response. Ghorbani et al. (2002) saw no effect when supplementing $10^{10}$ cfu/d *Propionibacterium,* but Stein et al. (2006) saw a positive response with $6\times10^{11}$ cfu/d *Propionibacterium*. Despite inconsistent effects on propionate production, no studies have detected increased plasma glucose levels in cows supplemented with LUB (West and Bernard, 2011; Boyd et al., 2011).

Fermentation of lactic acid to VFA by LUB may help to moderate rumen pH and mitigate bouts of acute acidosis. Acute acidosis is commonly characterized as below normal ruminal pH and high concentrations of lactic acid (Hernández et al., 2014). Acute acidosis is not very common in dairy cattle but may occur during substantial shifts in dietary starch content such as during the transition period. Research into DFM containing LUB has shown inconsistent outcomes on moderating rumen pH. Lawrence et al. (2021) found that *L. animalis* and *P. fruendenrichii* increased rumen pH during a dramatic shift in dietary starch content. When supplementing *M. elsdenii* in a highly fermentable diet, Kung and Hession (1995) saw increased pH in vitro whereas Aikman et al. (2011) saw no significant effect in vivo. These results may indicate that rumen pH moderation by LUB is dependent on bacterial species, diet, or research model.

Lactic acid utilizing bacteria are typically supplemented to ruminants due to their ability to consume and convert lactic acid to more favorable products. Fermentation of lactic acid may decrease lactate and increase propionate concentrations, therefore increasing rumen pH. These reactions may improve the ruminant's energy status and mitigate development of acute acidosis. Further research investigating the extent, mechanism, or bacterial interactions through which LUB affects ruminant performance may be warranted.

***Combination DFM***

Direct-fed microbials commonly include a combination of multiple strains of bacteria and/or yeast. This approach aims to optimize the benefits yeast and bacteria can provide individually, and together.

A prevalent combination is the yeast species *S. cerevisiae* with the LAB species *E. faciem*. Two transition cow studies reported increased milk and component concentrations when supplementing these combined species (Nocek et al., 2003; Nocek and Kautz, 2006). Additionally, these studies reported cows fed the DFM had increased plasma glucose and insulin concentrations as well as decreased BHBA and NEFA (Nocek et al., 2003; Nocek and Kautz, 2006). This may suggest supplemental species provided more substrate for gluconeogenesis, increasing available precursors for milk production and tissue use. In contrast, a similar transition cow study by AlZahal et al. (2014) saw no effect on milk production or plasma metabolites. When fed *S. cerevisiae* and *E. faciem*, starch ATTD was increased in lactating dairy cows (AlZahal et al., 2014), but not feedlot cattle (Beauchemin et al., 2003). DFM did not affect ATTD of organic matter, NDF, ADF, or protein in feedlot cattle (Beauchemin et al., 2003). Despite possible influences on nutrient digestibility or blood metabolites, this species combination did not affect BW or BCS in either dairy cows or feedlot steers (AlZahal et al., 2014; Nocek and Kautz, 2006; Beauchemin et al., 2003). Currently, no studies have reported on how this species may affect efficiency in ruminants.

Another common combination is *L. acidophilus* and *P. freudenreichii*, LAB and LUB species respectively. This combination increased milk yield and component concentrations in some studies (Boyd et al., 2011; West and Bernard, 2011), but not all (Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015). West and Bernard (2011) reported *L. acidophilus* and *E.*

*freudenreichii* increased efficiency (ECM/DMI), indicating the species may have improved diet digestibility or energy partitioning. Boyd et al. (2011) reported supplemented cows had increased NDF and CP ATTD, but other studies did not detect improvements in feed digestibility or efficiency (Ferraretto and Shaver, 2015; Boyd et al., 2011, Raeth-Knight et al., 2007). Studies found no effects of *L. acidophilus* and *P. freudenreichii* on BW, BCS, or plasma metabolite concentrations (Boyd et al., 2011; Ferraretto and Shaver, 2015; West and Bernard, 2011). The inconsistent results across these studies can likely be attributed to each using differing dosages or strains of *L. acidophilus* and *P. freudenreichii.*

Research of other species for DFM is much more limited. A study by Goetz et al. (2021) fed *C. beijerinckii* in combination with *P. kudriavzevii* to lactating dairy cattle. In this study, no effect was detected when considering all experimental animals but in a retrospective analysis, treatment effect differed based on original milk production. Treated animals who were low producers (<53kg/d) had increased ECM production whereas high producing cows (≥53kg/d) had decreased milk production. Xu et al. (2017) reported that a combination of *L. casei* and *L. planatarum* increased milk yield and decreased SCC after 15 days of supplementation but did not alter milk composition. *Propionibacterium* with *L. planatarum* or *L. rhamnosus* had no effects on milk yield or composition in both high and low starch diets (Philippeau et al., 2017). Lastly, in feedlot steers, *M. elsdenii* and *R. bromii* did not improve average daily gain or BCS change (Klieve et al., 2012). Due to the limited research of these microorganisms for DFM, little is known about their effects in ruminants.

Overall, DFM utilizing combinations of LAB, LUB, and yeast could benefit ruminants by leveraging the benefits of each species with their symbiotic interactions. Benefits may include

improved VFA production, blood metabolite concentrations, milk yield and composition, and overall animal efficiency.

## Endomicrobial Supplement

Many DFM contain species of bacteria and yeasts that are not native to the rumen of dairy cattle. This limits the organisms' ability to compete or interact with the established, stable rumen microbiome (Moraïs and Mizrahi, 2019). Weimer (2015) summarized several studies where rumen fibrolytic bacteria of donor species were supplemented to other, different ruminant species. In these studies, supplemented bacteria showed weak or no persistence in the recipient's rumen. This has generated interest in the efficacy of supplementing rumen species that are native to the recipient animal. The term "endomicrobial" has been used to describe DFM products containing microorganisms naturally occurring within the recipient animal species. The supplement studied in our research was an endomicrobial containing four, native rumen species of yeast and bacteria. These species of *Ruminococcus bovis*, *Butyrivibrios fibrisolvens*, *Clostridium beijerinckii*, and *Pichia kudriavzevii* have been proposed as promising microorganisms for endomicrobial supplementation.

### *Ruminococcus bovis*

*Ruminococcus bovis* is a novel bacteria species first isolated from the ruminal contents of a dairy cow and named in 2021 (Gaffney et al.). A part of the Firmicutes phylum, *R. bovis* is a strict anaerobic coccoid bacterium that grows in chains (Schoch et al., 2020; Gaffney et al., 2021). *R. bovis* can survive the rumen environmental conditions, but its optimal pH for growth is 7.0-7.5. Acidic conditions can hinder the bacteria's growth when between 6-6.5 pH and the species cannot grow when pH is lower than 5.5 (Gaffney et al., 2021).

*R. bovis* belongs to the *Ruminococcus* genus which comprises 3.9% of the total rumen bacterial community of cattle (Henderson et al., 2015). This genus is important for starch degradation, constituting 70-80% of starch hydrolyzing bacteria in barley fed cattle (Xia et al., 2015). *R. bovis* ferments starch, glucose, and glycogen, but not other sugar sources including glycerol or cellobiose (Gaffney et al., 2021). Fermentation by *R. bovis* produces acetate as a major product, and ethanol and glycerol as minor products (Gaffney et al., 2021). These fermentation characteristics indicate that supplemental *R. bovis* could promote digestion of starch and production of acetate to improve milk yield or composition. Due to its recent identification, there is currently no published research regarding *R. bovis* as a DFM for ruminants.

***Butyrivibrios fibrisolvens***

First isolated from the rumen of dairy cattle in the 1950s, *B. fibrisolvens* is a strictly anaerobic bacteria (Bryant and Small, 1955). This species is an active fiber, starch, and sugar fermenter but has shown little to no cellulose degradation capabilities (Sechovcová et al., 2019). The primary fermentation output of *B. fibrisolvens* is butyrate and it is considered the major butyrate producer of the rumen. (Stewart et al., 1997). In addition to carbohydrate fermentation, this species conducts polyunsaturated fatty acid biohydrogenation, particularly of linoleic acid (Kepler et al., 1966; Polan et al., 1964). Biohydrogenation by *B. fibrisolvens* produces the intermediate conjugated linoleic acid, a fatty acid desired for its potential health properties (Kritchevsky, 2000). With these characteristics in mind, suggested benefits of supplemental *B. fibrisolvens* are enhanced fiber digestion, increased energy status due to greater butyrate production, and improved fatty acid profile of milk.

*B. fibrisolvens* may also enhance the cow's immune system. To digest β-mannans present in hemicellulose, *B. fibrisolvens* produces the fibrolytic enzyme β-mannanase (Nakai et

al.,1993). Through the hydrolysis of β-mannan by this enzyme, mannan-oligosaccharides are released (MOS, Franco et al., 2004). There is little known about MOS exact function in animals but research by Ibuki et al. (2014) found MOS may play an important role in the immune system, particularly in the GIT. Ibuki et al. (2014) reported that pigs with intestinal inflammation fed supplemental MOS had decreased expression in the colon of pro-inflammatory factors TNF-α, IL-1β, and IL-17. In dairy cattle, somatic cell count is positively related to immune expression of TNF-α, IL-1β, and IL-17. If MOS moderated these factors it dairy cattle, it may decrease SCC (Oviedo-Boyso et al., 2007; Rainard et al., 2015, 2016). Tewoldebrhan et al. (2017) reported that low doses of supplemented β-mannanase (0.1% of DM) decreased SCC in lactating dairy cows with no effect on milk or component yield. Besides SCC, previous research in calves and dry cows found that supplementing MOS improved overall animal health, performance, and immune response to a viral challenge (Heinrichs et. al., 2003; Franklin et al., 2005). Considering MOS and β-mannanase's effect on animal health, research is needed regarding how supplementation of *B. fibrisolvens* may affect the expression of immune factors and the subsequent animal response.

The *Butyrivibrios* genus compose 4.1% of the total rumen microbial community, one of the most prevalent genera in cattle (Henderson et al., 2015). *Butyrivibrios* is classified as a main genus in dairy cattle, indicating the genus is heritable and a part of the core microbiome (Gonzalez-Recio et al., 2018). *B. fibrisolvens* concentrations can also be affected by diet composition. In a study conducted with beef cattle, when animals were switched from a diet containing a forage to concentration ratio of 100:0 to 60:40, *B. fibrisolvens* concentrations decreased 10-fold (Fernando et al., 2010). This research supports previous findings of a small study with dairy cattle by Mrázek et al. (2006), highlighting the important relationship the

bacterial species shares with dietary fiber content. Although a common species identified in the rumen, research could not be found investigating *B. fibrisolvens* as a DFM.

***Clostridium beijerinckii***

*Clostridium beijerinckii* is a common bacteria isolated from many sources beyond the rumen including feces and soil (Boone et al., 2001). The species is commonly used for industrial biofuel production due to its efficient butanol production from various biomass sources. *C. beijerinckii* can utilize sucrose, cellobiose, and starch for growth, butyric acid, and acetic acid production (Leschine, 2005). Increased production of the VFA by the rumen can improve the ruminant's energy status as well as milk yield and composition.

Beyond VFA production, *C. beijerinckii* may benefit the rumen environment and microflora through its efficient production of $H_2$ from carbohydrate degradation (Hoang et al., 2018). Hydrogen as either an ion ($H^+$) or dissolved gas ($H_2$) is a central regulator of rumen fermentation and end products through modulation of the rumen redox potential (Czerkawski, 1986). Increased concentrations of $H_2$ decreases rumen redox potential, promoting fermentation and production of propionate and methane. When partial pressure of rumen $H_2$ decreases, fermentation shifts and favors acetate production over other end products (Hegarty and Gerdes, 1999). The fermentation characteristics of *C. beijerinckii* suggests supplementation of the species may moderate the rumen's redox potential encouraging fermentation and VFA production.

*Clostridium beijerinckii* as a DFM has been scarcely researched. Recently, Goetz et al. (2021) fed a combination of *C. beijerinckii* and *P kudriavzevii* to lactating dairy cattle. In this study, no effect was detected when considering all experimental animals for milk yield and composition, DMI, efficiency or body weight. When conducting a retrospective analysis,

treatment had differing responses based on a cow's milk production levels. Cows fed DFM who were low producers (<53kg/d) experienced improved ECM production. In contrast, high producing cows (≥53kg/d) decreased in milk yield. Feeding the same species combination, Lefler et al. (2020) reported a treatment by week interaction with improved daily milk, ECM, and milk fat yield. These studies provide evidence that *C. beijerinckii* may be a beneficial DFM, but more research is needed to better understand its effects.

***Pichia kudriavzevii***

*Pichia kudriavzevii*, previously known as *Candida krusei* (Douglass et al., 2018), is the most abundant yeast in the rumen of dairy cattle (Fernandes et al., 2019). Named in 1965 by Boidin, Pignal, and Besson (1965), this species is widely abundant in nature and isolated from many sources including berries, feces, and eggs (Kurtzman et al., 2011). *P. kudriavzevii* is a very acid tolerant species, growing in conditions as low as 2 pH (Qvirist et al., 2017). In vitro experiments show that this yeast ferments a wide range of carbohydrates including glucose, sucrose, lactate, hemicellulose, cellulose, and starch (Kurtzman et al., 2011; Elahi and Rehman, 2018; Natalicia Mendes de Almeida et al., 2012). Due to its ability to break down cellulose and hemicellulose (Elahi and Rehman, 2018), *P. kudriavzevii* could improve rumen fiber digestibility. An in vitro study by Fernandes et al. (2019) found strains of *P. kudriavzevii* improved NDF digestibility at 12 and 48h of fermentation in rumen cultures. In contrast, Santos et al. (2015) reported a different strain of *P. kudriavzevii* decreased NDF digestibility at 12 and 24h of in vitro incubation. Together, these results indicate that *P. kudriavzevii* may benefit rumen fiber digestion, but responses may be strain dependent.

Interest in supplementing this yeast species to ruminants is relatively new and therefore, in vivo research is limited. As previously mentioned, research by Goetz et. al. (2021) found that

in combination with *C. beijerinckii*, treatment of these microorganisms increased ECM production in low producing cows (<54kg/d) but decreased milk production of high producing cows (>54kg/d). A study conducted by Intanoo et al. (2020) evaluated the detoxification effects of *P. kudriavzevii* supplementation in cows fed aflatoxin $B_1$ contaminated feeds. Results showed *P. kudriavzevii* had the capacity to detoxify aflatoxin $B_1$ and subsequently improve DMI and ECM yield in treated animals. Lastly, Suntara et al (2021) found that cows fed rice straw ensiled with *P. kudriavzevii* compared to *S. cerevisiae* had increased rumen microbe population 4h after feeding and improved DM digestibility. There was no difference between treatments on intake, milk production, or feed efficiency.

A common yeast in the rumen, *P. kudriavzevii* has recently piqued the interest of researchers as a potential species to use in DFM. With some promising results reported, more research is needed to properly understand the potential effects of *Pichia kudriavzevii* supplementation.

## Conclusions

The species colonizing the rumen are a diverse, abundant, and resilient community essential to animal performance and health. By supplementing combinations of bacteria and yeast, farmers may be able to improve rumen microbial fermentation, VFA production, and pH. Improvement of these factors can lead to enhanced cow energy status, health, efficiency, milk production and composition. Inconsistent results in research have indicated that dosage, species, strains, and diet are important considerations when developing DFM for dairy cattle.

The objective of our research was to investigate the combination of native rumen species *R. bovis*, *B fibrisolvens*, *C. beijerinckii*, and *P. kudriavzevii*'s effects on milk production and

composition, efficiency, blood metabolites, and nutrient digestion. These species have minimal reported research but display many beneficial functions, suggesting they may be good candidates for a DFM. This research will advance our knowledge of this novel combination of species, guide future research of best species and dosage for DFM, and determine the benefits this DFM may provide dairy farmers.

# CHAPTER 3
# MATERIALS AND METHODS

## Design and Treatments

Ninety lactating Holstein cows at the Michigan State University Dairy Cattle Teaching and Research Center were used in two cohorts (cohort 1, 11/2020 to 03/2021, n=39, 53% primiparous; and cohort 2, 01/2021 to 06/2021, n=51, 29% primiparous) in a randomized complete block design. Mean DIM, milk yield, and BW for all cows at the beginning of the study (mean ± SD) were 92 ± 23 d, 45.4 ± 10.3 kg/d, 659 ± 86 kg, respectively. Within cohort, cows were blocked by preliminary period parity, DIM, and ECM/$BW^{0.75}$, and within block, randomly assigned to treatment. All experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Cows were fed a common diet for a 14-d preliminary period, then treatments were topdressed daily on the common diets for 112 d. Treatments were 150 g of ground corn mixed with 1) no DFM (CON), 2) 5g of a live DFM (G2) containing *Clostridium beijerinckii* at $1.0\times10^7$ cfu and *Pichia kudriavzevii*, *Ruminococcus bovis,* and *Butyrivibrios fibrisolvens* at $1.0\times10^8$ cfu (Galaxis 2.0; Native Microbials Inc., San Diego, CA) or 3) 5g of a live DFM (G2P) that was similar to G2 but contained more *C. beijerinckii* ($7.5\times10^7$ cfu) and *P. kudriavzevii* ($1.0\times10^9$ cfu; Galaxis 2.0 Plus; Native Microbials Inc., San Diego, CA). Treatments were mixed into the top 15 cm of each cow's feed daily before she had access to it. Treatments were obtained from the manufacturer every 3 mo in individual, airtight daily packets, stored at 2°C, and mixed with the ground corn before feeding. The ingredient and nutrient composition of the diets fed as TMR are presented in Table 3.1.

Forage dry matter content was determined twice weekly, and diets were adjusted accordingly. Cows were milked three times daily at 730 h, 1530 h, and 2330 h (cohort 1, before

61 d of treatment) or 530 h, 1330 h, and 2130 h (cohort 1, after 61 d of treatment). Cohort 2 was milked at 400 h, 1200 h, and 2000 h. All cows were housed in tiestalls throughout the experiment and had access only to their own feed. Stalls were bedded with sawdust and cleaned three times daily. Orts were recorded daily prior to feeding. Feed was offered at 115% of expected intake once daily at 1030 h (cohort 1) or 800 h (cohort 2). Water was available ad libitum in each stall.

**Table 3.1. Ingredient and nutrient composition of common ration.**

| Ingredient | % of DM |
|---|---|
| Corn Silage | 29.3 |
| Alfalfa Silage | 13.6 |
| Ground Corn | 22.1 |
| Cottonseed, Whole | 6.8 |
| Soybean Meal | 8.5 |
| Soybean Hulls | 10.2 |
| Base Vitamin/Mineral Mix[1] | 2.0 |
| High Cow Supplement Mix[2] | 7.6 |
| Nutrient Composition | |
| DM[3] | 53.6 |
| NDF | 29.1 |
| Forage NDF | 17.7 |
| Starch | 27.3 |
| CP | 16.8 |
| RUP[4] | 33.9 |

[1]Vitamin and mineral mix contained 22.0% fine ground corn grain, 20.5% MIN-AD (MIN-AD Inc., Winnemucca, NV), 20.0% calcium carbonate, 19.1% calcium phosphate di, 10.0% sodium chloride, 4.6% sodium sesquinate, 2.0% selenium, and <1% of each of the following: bleachable fancy tallow, Intellibond VITAL 5 (Micronutrients USA LLC, Indianapolis, IN), vitamin E, vitamin A, and Vit D3 500 (Baltivet, Dubingai, Lithuania).
[2]High cow supplement mix contained 39.5% Amino Plus (Ag Processing Inc., Omaha, NE), 18.4% Caledonia Pass (Caledonia Farmers Elevator, Caledonia, MI), 15.8% sodium sesquicarbonate, 12.8% calcium carbonate, 8.7% fine ground corn grain, 2.7% urea, and 1.1% Smartamine M (Adisseo, Alpharetta, GA).
[3]Expressed as percent of as fed.
[4]Expressed as percent of CP based on 2021 NRC.

## Data Collection and Analysis

Body weight was measured for each cow 3 d per week. Body condition was scored by 3 trained investigators on a 5-point scale in 0.25 increments (Wildman et al., 1982) at -14, 0, 28, 56, 84, and 112 d of treatment. Daily milk yield was automatically recorded at each milking. Milk samples were collected for 6 consecutive milkings per week for component analysis. Samples were stored with preservative (Bronolab W-II liquid, Advanced Instruments, Norwood, MA) at 4°C until analysis. Individual milk samples were analyzed by CentralStar Cooperative, Inc (Grand Ledge, MI) for fat, true protein, lactose, MUN, and SCC concentrations by mid-infrared spectroscopy (AOAC, 1990, method 972.160). Milk yield and component concentrations for each milking were summed for a daily total and to calculate ECM and milk component yields. Energy-corrected milk was calculated as: ECM = [(0.324 x kg milk) + (12.816 x kg milk fat) + (7.129 x kg milk protein)].

Samples of all diet ingredients and TMR (~0.5 kg) were collected once weekly and stored at -20°C until composited by month and dried. Apparent total tract digestibility (ATTD) was determined on 10 blocks (30 cows) in each cohort. Samples of all diet ingredients (~0.5 kg) and orts from each cow (~1.0 kg) were collected daily over 5 d (d 29 to 34 for cohort 1 and d 35 to 40 for cohort 2). Within cohorts, samples of diet ingredients were composited for the 5 d and orts were composited by cow for the 5 d. Feces (~400 g) was sampled from the rectum of each cow or during defecation every 15 h resulting in 8 samples/cow representing every 3 h over a day. Feces were stored at -20°C until dried and composited on an equal DM basis for each cow. Diet ingredients, orts, and fecal samples were dried at 55°C for 72 h in a forced-air oven to determine DM. Dried samples were ground with a Wiley mill (5-mm screen; Arthur H. Thomas, Philadelphia, PA). Samples of diet ingredients, orts, and feces were analyzed by Cumberland

Valley Analytical Services (Waynesboro, PA) for CP (method 990.03; AOAC, 2000), starch (Hall, 2009), and ether extract (method 2003.05; AOAC, 2006). Ash was determined according to AOAC method 942.05 (2000) modified to ash a 1.5 g sample for 4 h. NDF and indigestible NDF were determined according to Van Soest et al. (1991) modified to use Whatman 934-AH glass micro-fiber filters with 1.5-µm particle retention (Cytiva, Marlborough, MA). Indigestible NDF, estimated as NDF residue after 240 h in vitro fermentation, was used as an internal marker to predict fecal output (Cochran et al., 1986).

Blood was sampled (~15 mL) at every fecal collection and on 93 ± 1 d at -1, +2, and +6 h after feeding into EDTA- and NaF-coated tubes. Plasma was harvested after centrifugation at 2,000 × *g* for 15 min at 4°C and stored at -20°C until composited by cow and analyzed. Plasma non-esterified fatty acid (NEFA), glucose, and insulin concentrations were analyzed using commercially available kits according to the manufacturer's instructions (NEFA: Sekishui Diagnostics, Burlington, MA; glucose: Sigma-Aldrich, St. Louis, MO; insulin: Mercodia, Uppsala, Sweden). Absorbance was measured with a micro-plate reader (SpectraMax 190; Molecular Devices Corp., Sunnyvale, CA).

In addition to ECM and ECM/DMI, other efficiency measurements were calculated for this study. Milk energy (MilkE), captured energy (CapE), and feed energy (FeedE), and IOFC were calculated and analyzed. Milk energy (Mcal/kg), an estimate of energy secreted in milk from fat, protein, and lactose, was calculated as: MilkE = [(9.29 x kg fat/kg milk) + (5.63 x kg true protein/kg milk) + (3.95 x kg lactose/kg milk)] (NASEM, 2021; equation 3-14b). Captured energy (Mcal/kg), an estimate of change in body tissue energy and energy secreted in milk, was calculated as: CapE = [MilkE + 6.3 x BW change/d] (NASEM, 2021). Energy content of diet (GE, Mcal/kg), an estimate of the energy content of offered feed, was calculated using NASEM

equation 3-2 (2021). Consumed feed energy (FeedE) was calculated as: FeedE= [Diet Energy x DMI]. IOFC was calculated as the difference between milk revenue and feed costs using average 2021 milk component prices (USDA, 2022a, 2022b).

## Statistical Analysis

All data were analyzed using the mixed model of SAS (version 9.4; SAS Institute, Cary, NC) according to the following model:

$$Y_{ijkl} = Cov + \mu + C_i + P_j + C_i \times P_j + B_k(C_i \times P_j) + T_l + T_l \times C_i + T_l \times P_j + T_l \times C_i \times P_j + e_{ijkl}$$

where $Y_{ijkl}$ = dependent variable, Cov = fixed effect of covariate, $\mu$ = overall mean, $C_i$ = fixed effect of cohort (i = 1 to 2), $P_j$ = fixed effect of parity (j = 1 to 2), $C_i \times P_j$ = interaction of cohort and parity, $B_k(C_i \times P_j)$ = random effect of block within cohort and parity, $T_l$ = fixed effect of treatment (l = 1 to 3), $T_l \times C_i$ = interaction of treatment and cohort, $T_l \times P_j$ = interaction of treatment and parity, $T_l \times C_i \times P_j$ = interaction of treatment, cohort, and parity, and $e_{ijkl}$ = residual error. BW change/d and BCS change/28 d were analyzed using the same model but excluding the covariate variable. Normality of results was tested using box plots, normal probability, and homogeneity of variances. SCC displayed non-normality so was log transformed (including covariate SCC data) for analysis, and back-transformed for presentation. Main effects were declared significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.15$. All data were expressed as LSM and SEM, unless otherwise specified. Preplanned contrasts were control vs DFM and G2 vs G2P.

# CHAPTER 4

## RESULTS AND DISCUSSION

Our study is the first to evaluate this combination of microorganisms supplemented to dairy cattle and the effect on milk production, digestibility, and blood metabolites. In our study, treatment did not alter yield of milk, protein, fat, lactose, MUN, or ECM (all $P > 0.4$, table 4.1). One of the proposed benefits of feeding DFM to cattle is improved milk yield and composition but responses have been variable. DFM containing *S. cerevisiae* and *E. faciem* improved milk yield (+1.20 kg/d) and protein concentration (+0.21%) in a study by Nocek et al. (2003) but not in a similar study by AlZahal et al. (2014). Similarly, a combination of *L. acidophilus* and *P. fruendenrechii* increased milk yield (2.4 kg/d, Boyd et al., 2011) and ECM (1.95 kg/d, West and Bernard, 2011) in some studies but not all (Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015). However, Goetz et al. (2021) detected no milk production or composition differences when feeding a DFM containing *C. beijerinckii* and *P kudriavzevii*, two of the species utilized in our study. When conducting a retrospective analysis, Goetz et al. (2021) reported cows who were low milk producers in the baseline period responded to treatment differently than high producing cows. When fed DFM, low producing cows (<53kg/d ECM) significantly increased ECM yield whereas high producing cows (>53kg/d ECM) tended to decrease milk yield. This indicates that responses to DFM supplementation, specifically *C. beijerinckii* and *P kudriavzevii*, may be affected by cow's milk production level. In our study, cows produced 44 kg of milk/d with 3.9% fat and 3.2% protein on average, and we observed no difference in treatment response for primiparous vs multiparous cows or by pretreatment milk yield. More experiments investigating differing responses to DFM supplementation between high and low producing cows may be warranted.

In our study, treatment significantly decreased SCC. Average SCC of CON was 20,700 cells/ml. Treatment decreased SCC ($P$=0.05; 7,200 cells/ml for G2 and 4,200 cells/ml for G2P), but G2 and G2P were not different ($P$>0.2). The cause for this improvement is unknown but may be connected to the potential role of *B. fibrisolvens* in immune modulation. *B. fibrisolvens* produces the enzyme β-mannanase, which hydrolyzes β-mannan present in hemicellulose (Nakai et al.,1993). This reaction releases mannan oligosaccharides (MOS), a molecule found to play an important role in moderating expression of cytokines including TNF-α, IL-1β, and IL-17 in gastrointestinal tissue (Franco et al., 2004; Ibuki et al., 2014). The expression of these proteins has been positively related to SCC, indicating MOS may be able to influence SCC through mediation of these cytokines (Oviedo-Boyso et al., 2007). Tewoldebrhan et al. (2017) reported low doses of supplemented β-mannanase (0.1% of DM) decreased SCC in lactating dairy cows with no effect on milk or component yield. Thus, I suggest *B. fibrisolvens* included in the treatment increased production of MOS through β-mannan hydrolysis, improved immune function, and decreased SCC. In our study, multiple species were supplemented and therefore, the SCC effect cannot be conclusively attributed to one organism. Additional research is required to understand the underlying relationship and mechanisms of DFM species and their effect on SCC.

**Table 4.1. Effects of DFM supplementation on milk yield, milk composition, BW, BCS, and efficiency.**

| | Treatment[1] | | | SEM | P-Value | |
|---|---|---|---|---|---|---|
| Variable | CON | G2 | G2P | | CON vs Trt | G2 vs G2P |
| DMI (kg) | 29.6 | 29.3 | 28.8 | 0.275 | 0.08 | 0.14 |
| Milk (kg/d) | 43.8 | 44.4 | 44.2 | 0.523 | 0.46 | 0.75 |
| ECM[2] (kg/d) | 461 | 45.9 | 45.7 | 0.512 | 0.60 | 0.73 |
| Fat (%) | 3.90 | 3.85 | 3.87 | 0.053 | 0.37 | 0.77 |
| Fat (kg/d) | 1.69 | 1.68 | 1.68 | 0.023 | 0.47 | 0.89 |
| Protein (%) | 3.21 | 3.19 | 3.19 | 0.017 | 0.23 | 0.88 |
| Protein (kg/d) | 1.41 | 1.40 | 1.39 | 0.018 | 0.58 | 0.52 |
| Lactose (%) | 4.92 | 4.93 | 4.92 | 0.007 | 0.37 | 0.47 |
| Lactose (kg/d) | 2.27 | 2.17 | 2.20 | 0.954 | 0.94 | 0.52 |
| MUN (mg/ml) | 13.0 | 13.0 | 12.9 | 0.019 | 0.89 | 0.57 |
| SCC[3] (x1,000 cells/ml) | 20.7 | 13.5 | 16.5 | 2.18 | 0.05 | 0.28 |
| Feed Efficiency (ECM/DMI) | 1.54 | 1.57 | 1.58 | 0.016 | 0.06 | 0.43 |
| Milk Energy (Mcal/kg) | 32.6 | 32.5 | 32.3 | 0.36 | 0.60 | 0.72 |
| Milk Energy/Feed Energy | 0.244 | 0.245 | 0.249 | 0.0022 | 0.17 | 0.30 |
| Captured Energy/Feed Energy | 0.267 | 0.266 | 0.269 | 0.0022 | 0.74 | 0.21 |
| BW (kg) | 694 | 687 | 686 | 2.47 | 0.02 | 0.61 |
| BW Change (kg/d) | 0.508 | 0.375 | 0.380 | 0.029 | 0.001 | 0.89 |
| BCS | 3.20 | 3.14 | 3.16 | 0.021 | 0.05 | 0.48 |
| BCS Change (unit/28d) | 0.070 | 0.052 | 0.048 | 0.008 | 0.04 | 0.74 |

[1] Treatment were 1) control (CON); 2) 5g of Galaxis 2.0 (G2; Native Microbials, Inc.); 3) 5g of Galaxis 2.0 Plus (G2P; Native Microbials, Inc).
[2] Energy-corrected milk; ECM = [(0.324 × kg milk) + (12.95 × kg milk fat) + (7.20 × kg milk protein)].
[3] Due to non-normality, SCC was log transformed for analysis. Means and SEM were back transformed for reporting.

In dairy cattle, milk yield is primarily determined by energy intake. Energy intake is determined by the diet's net energy content and the animal's intake of the diet, making DMI important for supporting milk production. In our study, average DMI of CON cows was 29.6 kg/d. Compared with CON, DMI tended to decrease with DFM treatments (*P*=0.08; G2= 29.3

kg/d, G2P=28.8 kg/d, SEM=0.275) with a trend for the difference between G2 and G2P (*P*=0.14). This response differs from that of Goetz et al. (2021), who found no effect on DMI from supplementing *C. beijerinckii* and *P. kudriavzevii*, two of the species used in our study. Ferraretto and Shaver (2015) reported a tendency for decreased DMI when cows were supplemented *P. freudenreichii* and *L. acidophilus*, although others did not detect DMI effects with the same species (Raeth-Knight et al., 2017; Boyd et al., 2011; West and Bernard, 2011). These species may increase rumen propionate concentrations (Raeth-Knight et al., 2017) and increased propionate concentrations have led to decreased meal size and subsequently, reduced DMI (Allen, 2000). None of the species supplemented in our study produce propionate but may have encouraged satiety through acetate and butyrate production. *R. bovis* and *C. beijerinkii* both produce acetate through the fermentation of starch. Past research has shown infusions of acetic acid can have hypophagic effects (Gauldrón-Duarte and Allen, 2018; Sheperd and Combs, 1998). Additionally, *B. fibrisolvens* and *C. beijericnkii* produce butyrate through fermentation. Forms of butyrate have decreased DMI in some studies (Urrutia et al., 2019; Simkins et al., 1965), but not all (Izumi et al., 2019). We speculate that DFM supplementation may have increased rumen concentrations of acetate and butyrate, consequently promoting satiety and decreasing DMI. Rumen VFA concentrations were not recorded in this study and therefore, follow up research using this particular DFM should quantify its effects on rumen VFA concentration.

Our study is the first to evaluate this combination of microorganisms supplemented to dairy cattle and the effect on digestibility. Digestibility plays an important role in DMI, energy partitioning, and efficiency. In our study, apparent total tract digestibility of organic matter (OM) tended (*P*=0.06) to decrease with treatment (CON=66.1%, G2=64.8%, G2P=65.6%,

SEM=0.004, table 4.2). A tendency for a difference between G2 and G2P was detected (*P*=0.15); this tendency for greater digestibility of OM in G2P than G2 could be related to the tendency for lower DMI in G2 than G2P. Decreased rumen digestibility of OM can increase rumen physical fill and retention, triggering satiety signals and decreasing the cow's desire to eat (Allen, 2000). In our study, only apparent total tract digestibility was measured so conclusions cannot be drawn on difference in sites of digestion and their subsequent effect on satiety and DMI. Based on the fermentation characteristics of the species supplemented in our study, improved digestibility, rather than decreased, would have been expected, specifically of starch and fiber. Average apparent total tract digestibilities for starch, NDF, CP, and fat were 98%, 45%, 60%, and 64%, respectively, and were not altered by our DFM treatments (P>0.15). Intanoo et al. (2020) observed similar results when feeding *P. kudriavzevii* to cows given an aflatoxin contaminated diet. Several studies evaluating other DFM species combinations have reported no effects on apparent total tract digestibility of starch, NDF, OM, CP, or ADF (Yoon and Stern, 1996; Philippeau et al., 2017; Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015; Boyd et al., 2011). Beauchemin et al. (2003) reported that feedlot cattle had decreased total tract digestibility of OM due to decreased ruminal digestion of ADF and intestinal digestion of fiber when receiving a DFM of *E. faciem* and *S. cerevisiae*. The reason for why endomicrobial supplementation decreased OM digestibility in our study is unclear and requires more investigation.

**Table 4.2. Effect of DFM supplementation on nutrient digestibility.**

| | Treatment[1] | | | SEM | P-Value | |
|---|---|---|---|---|---|---|
| Variable | CON | G2 | G2P | | CON vs Trt | G2 vs G2P |
| DMI (kg) | 29.6 | 29.3 | 28.8 | 0.28 | 0.08 | 0.14 |
| OM digestibility (%) | 66.1 | 64.8 | 65.6 | 0.44 | 0.06 | 0.15 |
| NDF digestibility (%) | 45.4 | 43.7 | 45.1 | 0.75 | 0.19 | 0.12 |
| Starch digestibility (%) | 98.5 | 98.5 | 98.4 | 0.18 | 0.88 | 0.66 |
| CP digestibility (%) | 60.8 | 59.8 | 60.6 | 0.75 | 0.49 | 0.48 |
| Fat digestibility (%) | 65.7 | 62.5 | 63.3 | 1.72 | 0.20 | 0.74 |

[1] Treatment were 1) control (CON); 2) 5g of Galaxis 2.0 (G2; Native Microbials, Inc.); 3) 5g of Galaxis 2.0 Plus (G2P; Native Microbials, Inc).

In our study, DFM treatments decreased BW change from 0.51 to 0.38 kg/d ($P$<0.0001, SEM=0.029, table 4.1) and BCS change from 0.7 to 0.05 BCS units/28d ($P$=0.04, SEM=0.008). No difference between G2 and G2P was detected ($P$>0.7). Our results differ from Goetz et al. (2021), who detected no difference in BW or BCS for cattle fed *C. beijerinckii* and *P. kudriavzevii*. Additionally, they noted a tendency for high producing cows (>53kg/d ECM) fed DFM to have increased BCS and suggested additional energy garnered from the supplement was partitioned to tissue. Many other studies evaluating DFM supplements report no effects of treatment on BW or BCS change (AlZahal et al., 2014; Beauchemin et al., 2003; Boyd et al., 2011; Ferreratto and Shaver, 2015; West and Bernard, 2011; Klieve et al., 2012). Body weight and body condition score are important indicators of animal energy status, milk production, and health (Roche et al., 2009). In our study, treated animals tended to consume less dry matter, have lower OM digestibility, and gain less weight and condition. Treated animals also maintained the same production of milk and ECM as control animals. This suggests treated animals may have partitioned more energy and nutrients away from tissue gain and toward milk production.

Although treatment tended to decrease DMI in our study, ECM was not affected. This resulted in a tendency for increased efficiency (ECM/DMI) in treated animals. Treatments tended to increase ECM/DMI from 1.54 to 1.57 (G2) or 1.58 (G2P; *P*=0.06, SEM=0.016, table 4.1) but with no difference between G2 and G2P. A similar study (Goetz et al., 2021) observed that supplementing *C. beijerinckii* and *P. kudriavzevii* increased ECM/DMI over time due to increased ECM and similar DMI. Similarly, West and Bernard (2011) saw cows supplemented with *L. acidophilus* and *P. fruendenreichii* had increased ECM/DMI due to increased ECM and no change in DMI. Increased ECM/DMI may indicate greater utilization and partitioning of feed energy through improved rumen conditions, function, or microbial yield. Efficiency responses are likely affected by factors such as dosage, diet, and animal's physiological state as other studies feeding the same species as West and Bernard (2011) reported no differences in efficiency measures (ECM/DMI or FCM/DMI) (Ferraretto and Shaver, 2015; Boyd et al., 2011; Raeth-Knight et al., 2007).

In our study, DFM did not significantly alter MilkE/FeedE or CapE/FeedE (*P*>0.2, table 4.1). Few other DFM studies have evaluated energy metrics to assess efficiency. Weiss et al. (2008) reported feeding *Propionibacterium* strain P169 to transition cows increased net energy of lactation use per unit of DMI despite no treatment effect on DMI or ECM/DMI. Supplementation of *S. cerevisiae* and *E. faciem* did not improve net energy balance in transition cows (AlZahal et al., 2014). Considering our results and others, it appears species of microorganisms or animal physiological state may affect energy efficiency.

Direct-fed microbials have been proposed to improve energy metabolism for dairy cattle. Blood markers including glucose, insulin, and NEFAs are indicators of energy metabolism and

were analyzed in our study. Our study is the first known research to investigate supplementing these microorganisms and their effect on blood metabolites.

Glucose is the main fuel source for dairy cattle and is critical to maintain good health and milk production. Propionate produced by the rumen microbiome are converted to glucose in the liver and thus, serve as the main glucose source for dairy cattle. Therefore, DFMs may increase glucose concentrations by improving the rumen microbiome's production of propionate. In our study, treatment did not significantly alter plasma glucose concentrations ($P$=0.3, table 4.3), similar to the results of many other DFM trials (Beauchemin et al., 2003; AlZahal et al., 2014; West and Bernard, 2011; Boyd et al., 2011; Francisco et al., 2002; Ghorbani et al., 2002). Two DFM studies in transition cows feeding *S. cerevisiae* and multiple strains of *Enterococcus* bacteria reported increased plasma glucose concentrations following parturition (Nocek et al., 2003; Nocek and Kautz, 2006).

Insulin, the essential regulator of blood glucose levels, is an equally important indicator of energy metabolism. In our study, plasma insulin concentrations tended to decrease by 11% with treatment ($P$=0.06; CON=0.95 μg/L, G2=0.81 μg/L, G2P=0.85 μg/L, SEM=0.057), but no difference between G2 and G2P was detected ($P$=0.6). This differs from the results of two transition cow studies by Francisco et al. (2002) and Nocek et al. (2003). Francisco et al. (2002) reported supplementing *Propionibacteria* to transition cows did not alter plasma glucose or insulin concentrations and suggested dosage or frequency of the DFM may have been inadequate. In comparison, when Nocek et al. (2003) fed *S. cerevisiae* and 2 *Enterococcus* strains pre- and post-partum, treated animals had increased plasma glucose and insulin concentrations. Nocek et al. (2003) suggests treated cows derived more energy from dietary carbohydrates to improve energy status.

Insulin and glucose are interdependent. Therefore, we would expect if plasma insulin concentrations increased, it is in response to increased glucose concentrations. In our study, no change was detected in plasma glucose concentrations although insulin concentrations decreased. This may indicate treatment resulted in altered tissue insulin response such as increased insulin sensitivity. We did not conduct tests to measure insulin sensitivity and therefore, cannot conclude how this DFM affected insulin sensitivity or responsiveness in treated animals. The tendency for decreased plasma insulin concentrations in treated animals likely did not substantially alter glucose uptake as 84% of glucose uptake is insulin independent (De Koster and Opsomer, 2013). Future research is required to understand more fully how this DFM affects insulin and glucose uptake in dairy cattle.

Non-esterified fatty acids are commonly released into the blood when dairy cattle mobilize adipose, usually to meet energetic requirements. Elevated plasma NEFA concentrations can indicate that cows are in negative energy balance, therefore, effects of DFM supplementation on NEFA concentrations are expected to be more pronounced during the transition period than in mid- and late-lactation cows (McArt et al., 2013). At the start of our study, all cows were greater than 55 DIM. Compared to CON, treatments did not significantly affect plasma NEFA concentration ($P$=0.4) but a difference was detected between G2 and G2P ($P$=0.03; Con=90 μM, G2=81 μM, G2P=92 μM, SEM=3.489). This is similar to results by Alzahal et al. (2014) and Nocek and Kautz (2006) who both reported no effect of DFM treatment in transition cows. In contrast, two studies in postpartum dairy cows reported decreased plasma NEFA concentrations in treated cows over time (Nocek et al., 2003; Francisco et al., 2002).

Compared to control, treatment did not affect plasma NEFA concentration, but there was a significant difference between G2 and G2P. When compared to G2P, G2 treated cows had

12% lower plasma NEFA concentrations (G2=81 μM, G2P=92 μM). G2 contained a lower concentration of *C. beijerinckii* and *P. kudriavzevii*, indicating the dosage of these species may affect fat mobilization, but the underlying mechanisms are unclear. Plasma insulin and NEFAs generally share an antagonistic relationship. Increased levels of plasma NEFA lowers insulin secretion and glucose uptake (Hue and Taegtmeyer, 2009). For this reason, it was unexpected that treatment tended to have lower plasma insulin concentrations when compared to control as well as treatment G2 having significantly lower plasma NEFA concentrations compared to G2P. The explanation for decreased plasma NEFA and insulin concentrations in G2 animals in unknown. Overall, plasma metabolite concentrations in our study indicate that treatment may have affected energy metabolism but requires more focused research to determine how and through what mechanisms.

**Table 4.3. Effect of DFM supplementation on plasma metabolite concentrations.**

| | Treatment[1] | | | SEM | P-Value | |
|---|---|---|---|---|---|---|
| Variable | CON | G2 | G2P | | CON vs Trt | G2 vs G2P |
| Glucose (md/dL) | 55.5 | 54.6 | 54.5 | 0.790 | 0.34 | 0.91 |
| NEFA (μM) | 90.4 | 81.2 | 92.4 | 3.49 | 0.41 | 0.031 |
| Insulin (μg/L) | 0.947 | 0.812 | 0.851 | 0.057 | 0.057 | 0.570 |

[1] Treatment were 1) control (CON); 2) 5g of Galaxis 2.0 (G2; Native Microbials, Inc.); 3) 5g of Galaxis 2.0 Plus (G2P; Native Microbials, Inc).

In summary, I found that supplementing post-peak dairy cows with a DFM containing *Clostridium beijerinckii, Pichia kudriavzevii, Ruminococcus bovis,* and *Butyrivibrios fibrisolvens* did not significantly alter milk yield or composition but significantly decreased SCC. Treated animals had significantly lower change in BW (0.13 kg/d less) and BCS (0.02 units/28d less) as well as a tendency for decreased DMI, leading to a tendency for improved efficiency. Additionally, treatment cows tended to have decreased plasma insulin concentrations with maintained glucose and NEFA concentrations, indicating treatment may have altered tissue

insulin response but further investigation using an insulin sensitivity test is required. Overall, DFM supplementation with these microorganism species did not notably improve performance or efficiency of lactating dairy cows. Different dosage of C. beijerinckii and P. kudriavzevii also did not significantly affect the performance outcomes of treated animals.

# CHAPTER 5

# OVERALL CONCLUSIONS

Direct-fed microbials have been used by the dairy industry to promote milk production and efficiency. Most organisms in commercial DFM are not native to the rumen, which may limit their ability to interact with the native microbiome (Weimer, 2015). Using species native to the dairy cow rumen may allow for improved interaction and manipulation of the rumen microbiome. Few studies have investigated the use of native rumen organisms in DFMs and their effects. The objective of this thesis was to evaluate DFM products containing organisms native to the dairy cow rumen and their effects on milk production and efficiency. This study examined a DFM containing *Clostridium beijerinckii, Pichia kudriavzevii, Ruminococcus bovis,* and *Butyrivibrios fibrisolvens* on milk production and efficiency of dairy cows.

In this research, DFM did not significantly alter milk yield or composition but significantly decreased SCC. DFM significantly lowered change in BW and BCS as well as tended to decrease DMI and improve milk/feed. We suggest that the reduced gains in BW and BCS of cows fed native DFM indicate that these treatments caused cows to partition energy differently than control cows. Treated cows also tended to eat less and digest OM less efficiently but produce as much milk, which might explain the lower weight gain. Additionally, DFM did not alter plasma NEFA concentrations indicating fat mobilization was not altered to sustain production. The lack of effect on milk production and composition in this study is similar to a study by Goetz et al. (2021) who fed two of the same species as this study, *C. beijerinckii* and *P. kudriavzevii*, and also saw no change in production.

This study is the first to evaluate nutrient digestibility with these supplemented species. The organisms *R. bovis* and *C. beijerinckii* were expected to enhance starch digestion (Gaffney et al., 2021; Leschine, 2005). Digestibilities of several nutrients (NDF, starch, CP, fat) were not

improved by our DFM treatments, similar to studies of other DFM products (Yoon and Stern, 1996; Raeth-Knight et al., 2007; Ferraretto and Shaver, 2015; Philippeau et al., 2017). Perhaps the reason we saw no improvement in digestion or production from native DFM in our study was because our diet was high in starch (29%) that was from highly digestible sources (98.5% diet starch digestibility). Thus, further improvements in starch digestion were unlikely. Results might have been different if our basal diet had a lower starch digestibility.

In conclusion, these results indicate supplementation of this native rumen DFM does not significantly improve milk production or efficiency. This work provides foundational in vivo research for evaluation of native rumen species for use as a DFM. Further research is needed to identify how this DFM affects rumen microbiome dynamics, energy partitioning, with diets containing different amounts or sources of starch, and between high and low producing cows.

# APPENDIX

# APPENDIX

## Income Over Feed Cost

Income over feed cost (IOFC) estimates the economic outcomes of changes in DMI, ration cost, and milk production and composition. In our study, treatment did not significantly alter IOFC ($P$=0.7) indicating that although there was a tendency for decreased DMI in treated animals, this did not translate to a significant economic benefit using our initial prices. The IOFC for CON at baseline daily milk income and feed costs was $8.04 (Table A.1). Therefore, DFM cows would need to achieve a baseline IOFC of $8.04 to break-even and $8.14 to achieve a 2:1 return on investment when compared to the control animals. The average performance of DFM supplemented cows in our study did not break-even or achieve a 2:1 return, earning −$0.01/d less than CON (G2P baseline IOFC=8.03) (Table A.2).

I then conducted a sensitivity analysis to determine how shifts in milk prices and feed costs would affect the IOFC of CON and G2P as well as the difference between them (Table A.3). In this sensitivity analysis, IOFC was calculated using individual treatment least-squares means for daily milk income and feed costs of CON and G2P. IOFC was calculated as the difference between daily milk income and feed costs. Daily milk income was calculated as: Daily Milk Income = [Milk Price * (Milk lbs/100)]. Milk Price was calculated as: Milk Price = [(Fat% * $/lb of Butterfat) + (Protein% * $/lb of Protein) + (5.73 * $/lb Other Solids) + PPD]. The milk price was calculated using average 2021 milk component prices (USDA, 2022a, 2022b). I assumed the DFM would cost $0.10/cow/d (Torres, 2021) and average feed costs were $0.15 per lb of dry matter (Liu et al., 2021). Therefore, daily feed cost for control animals was calculated as: Daily Feed Cost = [DMI lbs * $0.15]. For cows receiving the DFM treatments: Daily Feed Cost = [(DMI lbs * $0.15) + $0.10].

Milk income and feed costs were decreased or increased by 15% or 30% from baseline and IOFC was recalculated. As expected, IOFC was greatest for both CON and G2P when milk income was increased 30% and feed costs decreased 30% (CON=$16.33, G2P=$16.18). Additionally, IOFC for both treatments were lowest when milk income was decreased 30% and feed costs increased 30% (CON= −$0.25, G2P= −$0.20). As milk income decreased and feed cost increased, G2P garnered greater IOFC compared to CON but still did not achieve a 2:1 return. G2P generated the most additional IOFC compared to CON when milk income decreased 30% and feed costs increased 30% (difference = $0.15). In contrast, as milk income increased and feed costs decreased, G2P generated even less IOFC compared to CON. When milk income increased 30% and feed costs decreased 30%, G2P generated the least IOFC compared to CON (difference = −$0.16). Overall, the DFM supplemented in this trial did not significantly alter IOFC compared to CON or achieve a 2:1 return on investment not only at baseline but also when milk income and feed costs increased or decreased.

**Table A.1. Income over feed costs when milk income or feed costs change for control treatment.**

| | | Change in Milk Income ($/d)[1] | | | | |
|---|---|---|---|---|---|---|
| | | **−30%** | **−15%** | **0%** | **+15%** | **+30%** |
| **Change in Feed Costs[2]** | **+30%** | −0.25 | 2.42 | 5.10 | 7.78 | 10.45 |
| | **+15%** | 1.22 | 3.89 | 6.57 | 9.25 | 11.92 |
| | **0%** | 2.69 | 5.36 | 8.04 | 10.72 | 13.39 |
| | **−15%** | 4.16 | 6.83 | 9.51 | 12.19 | 14.86 |
| | **−30%** | 5.63 | 8.30 | 10.98 | 13.66 | 16.33 |

[1]Baseline milk income for control was $17.84.

[2]Baseline feed costs for control was $9.80.

**Table A.2. Income over feed costs when milk income or feed costs change for G2P treatment.**

| | | Change in Milk Income ($/d)[1] | | | | |
|---|---|---|---|---|---|---|
| | | **−30%** | **−15%** | **0%** | **+15%** | **+30%** |
| **Change in Feed Costs[2]** | **+30%** | −0.20 | 2.45 | 5.10 | 7.74 | 10.39 |
| | **+15%** | 1.31 | 3.96 | 6.60 | 9.25 | 11.90 |
| | **0%** | 2.74 | 5.38 | 8.03 | 10.68 | 13.32 |
| | **−15%** | 4.16 | 6.81 | 9.46 | 12.10 | 14.75 |
| | **−30%** | 5.59 | 8.24 | 10.88 | 13.53 | 16.18 |

[1]Baseline milk income for G2P was $17.64.

[2]Baseline feed costs for G2P was $9.61.

**Table A.3. Difference between G2P and CON income over feed costs when milk income or feed costs change[1].**

| | | Change in Milk Income ($/d)[2] | | | | |
|---|---|---|---|---|---|---|
| | | **−30%** | **−15%** | **0%** | **+15%** | **+30%** |
| **Change in Feed Costs[3]** | **+30%** | 0.15 | 0.12 | 0.09 | 0.06 | 0.03 |
| | **+15%** | 0.09 | 0.06 | 0.03 | 0.00 | −0.03 |
| | **0%** | 0.05 | 0.02 | −0.01 | −0.04 | −0.07 |
| | **−15%** | 0.01 | −0.02 | −0.05 | −0.08 | −0.11 |
| | **−30%** | −0.04 | −0.07 | −0.10 | −0.13 | −0.16 |

[1]Calculated as: Difference = [G2P IOFC – CON IOFC].

[2]Baseline milk income for treatments were Con = $17.84 and G2P = $17.64.

[3]Baseline feed costs for treatments were Con = $9.80 and G2P = $9.61.

## Differences Between Cohorts

Due to barn space and animal availability, cows in this study were divided into two cohorts. Cohort 1 (39 cows) completed the experiment from 11/13/2020 to 3/19/2021 and Cohort 2 (51 cows) from 1/29/2021 to 6/4/2021. During statistical analysis, we discovered significant differences in average production and efficiency variables between the two cohorts (Table A.4). Also, a significant interaction of cohort by treatment was identified for BW change/d ($P$=0.02). There was tendency for the interaction of cohort by treatment for milk fat concentration, ECM/DMI, MilkE/FeedE, CapE/FeedE, BW, and plasma insulin concentration (all $0.05<P<0.015$, table A.5). Due to the differences between cohorts and the interaction of cohort by treatment, we decided to investigate what may have been contributing factors.

The characteristics of animals comprising the two cohorts differed. Cohort 1 was composed of 54% primiparous cows whereas cohort 2 was 29% primiparous cows. Primiparous cows typically produce less milk than multiparous cows (Vijayakumar et al., 2017) and may have contributed to the difference in overall milk yield and composition between cohorts. Compared to cohort 2, cohort 1 had lower average milk production at the beginning of trial (40 kg/d vs 49 kg/d), but higher average milk yield of the entire treatment period (45.6 kg/d vs 42.7 kg/d). This may indicate that cohort 1 cows had higher peak milk or were more persistent than cohort 2 cows. Average DIM at the beginning of the trial were similar between the cohorts (Cohort 1=92 DIM, Cohort 2= 93 DIM) and likely did not contribute to overall differences.

Diet is a key factor contributing to the performance of dairy cows. Feed ingredients used in dairy diets, particularly forages, can vary in nutrient composition and digestibility over time. Due to this, we evaluated the nutrient composition of monthly TMR samples to identify any differences between the diets each cohort received. The average CP, NDF, and fat content of the

TMR was similar between cohorts (Table A.6). The dry matter content of Cohort 1's TMR was slightly lower than Cohorts 2's (52.7% vs 54.3%). Additionally, cohort 1's TMR had slightly lower content of starch than cohort 2's (26.9% vs 27.7%) but this starch was more digestible in-vitro (7h in-vitro digestibility, 60.3% vs 57.3%). Apparent total-tract digestibility of starch in cohort 2 was higher than cohort 1 (98.8% vs 98.0%). Therefore, cohort 2 likely received more dietary digestible energy from starch than cohort 1.

Weather and environmental conditions can affect cow productivity. Low or high temperatures, as well as dramatic temperature shifts, can play a role in animal comfort and productivity (Zimbelman et al., 2009). Cohort 1 completed the experiment from Nov. to Mar. and Cohort 2 from Jan. to Jun. The average daily temperature for cohort 1 and 2 was −1°C and 6°C, respectively (Figure A.1). Cohort 2 had a greater range in average daily temperatures (−15°C to 24°C) compared to cohort 1 (−15°C to 14°C). Cohorts were also housed in two different barns. Consequently, there may have been unidentified barn environmental differences that affected cow productivity. Thus, exposure to different temperature and environmental conditions could be partly responsible for overall cohort performance differences.

Overall differences between cohorts for average milk yield, composition, and efficiency can likely be attributed to many factors including parity distribution, diet starch concentration and digestibility, and environmental conditions.

**Table A.4. Comparison of production, efficiency, digestibility, and plasma metabolite concentrations between cohorts.**

| Variable | Cohort[1] | | SEM | P-Value | |
|---|---|---|---|---|---|
| | 1 | 2 | | Cohort*Trt | Cohort 1 vs 2 |
| Milk Yield and Composition | | | | | |
| DMI (kg) | 29.6 | 28.8 | 0.266 | 0.933 | 0.030 |
| Milk (kg/d) | 45.6 | 42.7 | 0.490 | 0.797 | <0.001 |
| ECM[2] (kg/d) | 46.8 | 45.0 | 0.478 | 0.214 | 0.011 |
| Fat (%) | 3.78 | 3.97 | 0.032 | 0.530 | <0.001 |
| Fat (kg/d) | 1.68 | 1.69 | 0.017 | 0.196 | <0.001 |
| Protein (%) | 3.26 | 3.14 | 0.016 | 0.071 | <0.001 |
| Protein (kg/d) | 1.46 | 1.34 | 0.018 | 0.196 | <0.001 |
| Lactose (%) | 4.93 | 4.92 | 0.001 | 0.869 | 0.742 |
| Lactose (kg/d) | 2.24 | 2.10 | 0.026 | 0.637 | 0.001 |
| MUN (mg/ml) | 11.4 | 14.6 | 0.227 | 0.248 | <0.001 |
| SCC[3] (x1,000 cells/ml) | 16.3 | 17.7 | 1.80 | 0.905 | 0.564 |
| Efficiency | | | | | |
| Feed Efficiency (ECM/DMI) | 1.59 | 1.54 | 0.016 | 0.1369 | 0.0536 |
| MilkE/FeedE | 0.25 | 0.25 | 0.002 | 0.0388 | 0.5535 |
| CapE/FeedE | 0.27 | 0.27 | 0.002 | 0.0181 | 0.868 |
| Nutrient Digestibility | | | | | |
| OM digestibility (%) | 64.6 | 66.4 | 0.50 | 0.8884 | 0.010 |
| NDF digestibility (%) | 41.6 | 47.9 | 0.80 | 0.1852 | <0.001 |
| Starch digestibility (%) | 98.0 | 98.8 | 0.10 | 0.6216 | 0.002 |
| CP digestibility (%) | 60.7 | 60.1 | 0.60 | 0.4335 | 0.479 |
| Fat digestibility (%) | 61.2 | 66.5 | 1.40 | 0.7432 | 0.017 |
| BW and BCS | | | | | |
| BW (kg) | 689 | 688 | 2.04 | 0.052 | 0.692 |
| BW Change (kg/d) | 0.378 | 0.464 | 0.024 | 0.018 | 0.016 |
| BCS | 3.21 | 3.13 | 0.020 | 0.563 | 0.014 |
| BCS Change (unit/28d) | 0.071 | 0.042 | 0.006 | 0.449 | 0.003 |
| Plasma Metabolite Concentrations | | | | | |
| Glucose (md/dL) | 54.8 | 54.9 | 0.692 | 0.701 | 0.962 |
| NEFA (μM) | 87.1 | 88.8 | 2.942 | 0.191 | 0.680 |
| Insulin (μg/L) | 0.877 | 0.863 | 0.061 | 0.070 | 0.867 |

[1]Cohort 1 completed the trial from 11/13/2020 to 3/19/2021, cohort 2 completed the trial from 1/29/2021 to 6/4/2021.

[2] Energy-corrected milk; ECM = [(0.324 × kg milk) + (12.95 × kg milk fat) + (7.20 × kg milk protein)].

**Table A.4. (cont.)**

[3]Due to non-normality, SCC was log transformed for analysis. Means were back transformed for reporting. SEM were calculated as the difference between back transformed means and back transformed means ± SEM.

**Table A.5. Comparison of response to DFM supplementation broken out by cohort[1] and treatment[2].**

| Variable | Cohort 1 | | | SEM | Cohort 2 | | | SEM |
|---|---|---|---|---|---|---|---|---|
| | CON | G2 | G2P | | CON | G2 | G2P | |
| Milk Yield and Composition | | | | | | | | |
| DMI (kg) | 30.1 | 29.7 | 29.2 | 0.42 | 29.1 | 29.0 | 28.4 | 0.37 |
| Milk (kg/d) | 45.0 | 45.8 | 45.9 | 0.79 | 42.6 | 43.0 | 42.5 | 0.72 |
| ECM[3] (kg/d) | 46.5 | 46.5 | 47.3 | 0.77 | 45.7 | 45.3 | 44.1 | 0.70 |
| Fat (%) | 3.79 | 3.73 | 3.81 | 0.056 | 4.01 | 3.97 | 3.93 | 0.052 |
| Fat (kg/d) | 1.67 | 1.66 | 1.70 | 0.030 | 1.72 | 1.70 | 1.65 | 0.027 |
| Protein (%) | 3.26 | 3.24 | 3.28 | 0.025 | 3.17 | 3.15 | 3.10 | 0.024 |
| Protein (kg/d) | 1.45 | 1.46 | 1.48 | 0.027 | 1.36 | 1.35 | 1.30 | 0.025 |
| Lactose (%) | 4.92 | 4.94 | 4.93 | 0.011 | 4.92 | 4.93 | 4.92 | 0.010 |
| Lactose (kg/d) | 2.22 | 2.25 | 2.24 | 0.042 | 2.12 | 2.12 | 2.07 | 0.040 |
| MUN (mg/ml) | 11.3 | 11.7 | 11.2 | 0.32 | 14.7 | 14.4 | 14.6 | 0.28 |
| SCC[4] (x1,000 cells/ml) | 19.9 | 12.8 | 16.9 | 3.07 | 21.7 | 15.1 | 16.9 | 3.20 |
| Efficiency | | | | | | | | |
| Feed Efficiency (ECM/DMI) | 1.55 | 1.59 | 1.63 | 0.023 | 1.54 | 1.54 | 1.54 | 0.022 |
| MilkE/FeedE | 0.24 | 0.25 | 0.26 | 0.004 | 0.24 | 0.24 | 0.24 | 0.003 |
| CapE/FeedE | 0.27 | 0.27 | 0.28 | 0.004 | 0.26 | 0.27 | 0.26 | 0.004 |
| Nutrient Digestibility | | | | | | | | |
| OM digestibility (%) | 65.2 | 64.0 | 64.6 | 0.61 | 67.1 | 35.6 | 66.6 | 0.65 |
| NDF digestibility (%) | 42.4 | 41.2 | 41.0 | 1.02 | 48.4 | 46.1 | 49.2 | 1.09 |
| Starch digestibility (%) | 98.2 | 98.0 | 97.9 | 0.24 | 98.7 | 98.9 | 98.8 | 0.26 |
| CP digestibility (%) | 60.8 | 59.6 | 61.7 | 1.02 | 60.8 | 60.0 | 59.5 | 1.09 |
| Fat digestibility (%) | 62.0 | 60.0 | 61.6 | 2.35 | 69.3 | 65.1 | 65.1 | 2.51 |
| BW and BCS | | | | | | | | |
| BW (kg) | 693 | 684 | 691 | 3.55 | 693 | 691 | 680 | 3.34 |
| BW Change (kg/d) | 0.50 | 0.26 | 0.37 | 0.042 | 0.51 | 0.49 | 0.39 | 0.041 |
| BCS | 3.22 | 3.18 | 3.21 | 0.031 | 3.18 | 3.11 | 3.11 | 0.030 |
| BCS Change (unit/28d) | 0.09 | 0.06 | 0.07 | 0.011 | 0.05 | 0.04 | 0.03 | 0.011 |
| Plasma Metabolite Concentrations | | | | | | | | |
| Glucose (md/dL) | 55.5 | 55.0 | 54.0 | 1.08 | 55.4 | 54.2 | 55.0 | 1.15 |
| NEFA (μM) | 88.7 | 85.3 | 87.4 | 4.77 | 92.1 | 77.1 | 97.4 | 5.10 |
| Insulin (μg/L) | 1.05 | 0.76 | 0.82 | 0.079 | 0.85 | 0.86 | 0.88 | 0.084 |

[1]Cohort 1 completed the trial from 11/13/2020 to 3/19/2021, cohort 2 completed the trial from 1/29/2021 to 6/4/2021.

[2] Treatment were 1) control (CON); 2) 5g of Galaxis 2.0 (G2; Native Microbials, Inc.); 3) 5g of Galaxis 2.0 Plus (G2P; Native Microbials, Inc).

**Table A.5. (cont.)**

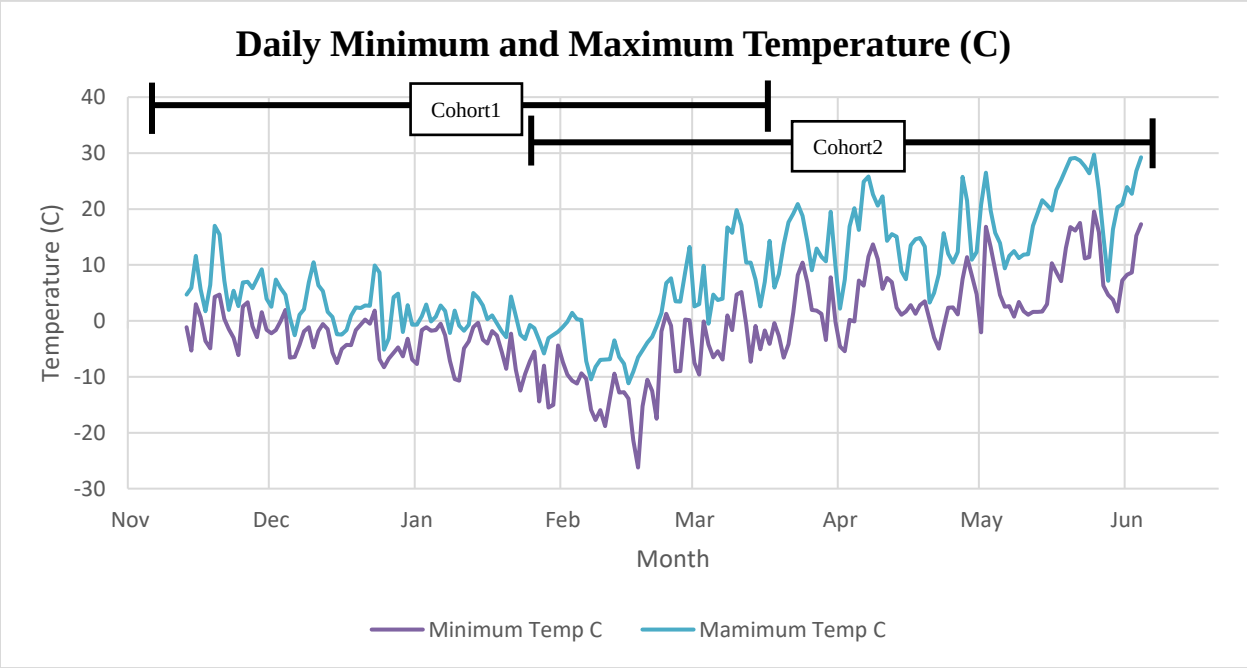
[3] Energy-corrected milk; ECM = [(0.324 × kg milk) + (12.95 × kg milk fat) + (7.20 × kg milk protein)].
[4]Due to non-normality, SCC was log transformed for analysis. Means were back transformed for reporting. SEM were calculated as the difference between back transformed means and back transformed means ± SEM.

**Table A.6. Nutrient composition of common ration for each cohort.**

| | Cohort[1] | |
|---|---|---|
| Nutrient, % of DM | 1 | 2 |
| DM[2] | 52.7 | 54.3 |
| CP | 16.8 | 16.8 |
| NDF | 29.2 | 28.9 |
| Starch | 26.9 | 27.7 |
| IV Starch Digestibility[3] | 60.3 | 57.3 |
| Fat | 3.5 | 3.3 |

[1]Cohort 1 completed the trial from 11/13/2020 to 3/19/2021, cohort 2 completed the trial from 1/29/2021 to 6/4/2021.
[2]Expressed as a percent of as fed.
[3]In vitro starch digestibility measured after 7 h of fermentation, expressed as a percent of starch.

**Figure A.1. Daily minimum and maximum temperatures for duration of trial.**



**REFERENCES**

## REFERENCES


Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., & Yáñez-Ruiz, D. R. (2014). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Animal Production Science*, *54*(9), 1449–1454. doi:10.1071/AN14337

Aikman, P. C., Henning, P. H., Humphries, D. J., & Horn, C. H. (2011). Rumen pH and fermentation characteristics in dairy cows supplemented with *Megasphaera elsdenii* NCIMB 41125 in early lactation. *Journal of Dairy Science*, *94*(6), 2840–2849. doi:10.3168/jds.2010-3783

Allen, M. S. (2000). Effects of diet on short-term regulation of feed intake by lactating dairy cattle. *Journal of Dairy Science*, *83*(7), 1598–1624. doi:10.3168/jds.S0022-0302(00)75030-2

AlZahal, O., McGill, H., Kleinberg, A., Holliday, J. I., Hindrichsen, I. K., Duffield, T. F., & McBride, B. W. (2014). Use of a direct-fed microbial product as a supplement during the transition period in dairy cattle. *Journal of Dairy Science*, *97*(11), 7102–7114. doi:10.3168/jds.2014-8248

AOAC. (2000a). Ash of Animal Feed (942.05). *Official Methods of Analysis*.

AOAC. (2000b). Protein (Crude) in Animal Feed, Combustion Method (990.03). *Official Methods of Analysis of AOAC International*.

AOAC. (2006). Crude Fat in Feeds, Cereal Grains, and Forages (2003.05). *Official Methods of Analysis*.

Bach, A., Iglesias, C., & Devant, M. (2007). Daily rumen pH pattern of loose-housed dairy cattle as affected by feeding pattern and live yeast supplementation. *Animal Feed Science and Technology*, *136*(1–2), 146–153. doi:10.1016/j.anifeedsci.2006.09.011

Ban, Y., & Guan, L. L. (2021). Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health. *Journal of Animal Science and Biotechnology*, *12*(1), 109. doi:10.1186/s40104-021-00630-x

Beauchemin, K. A., Yang, W. Z., Morgavi, D. P., Ghorbani, G. R., Kautz, W., & Leedle, J. A. Z. (2003). Effects of bacterial direct-fed microbials and yeast on site and extent of digestion, blood chemistry, and subclinical ruminal acidosis in feedlot cattle. *J. Anim. Sci, 81,* 1628-1640. https://academic.oup.com/jas/article/81/6/1628/4790374

Belanche, A., Kingston-Smith, A. H., Griffith, G. W., & Newbold, C. J. (2019). A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. *Frontiers in Microbiology*, *10*(FEB). doi:10.3389/fmicb.2019.00122

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews*, *70*(2).

Boidin, Pignal, & Besson. (1965). *Pichia kudriavzevii*. *Bulletin de La Société Mycologique de France*, *81*(589).

Boone, D., Castenholz, R., & Garrity, G. (2001). *Bergey's manual of systematic bacteriology*. Springer.

Boyd, J., West, J. W., & Bernard, J. K. (2011). Effects of the addition of direct-fed microbials and glycerol to the diet of lactating dairy cows on milk yield and apparent efficiency of yield. *Journal of Dairy Science*, *94*(9), 4616–4622. doi:10.3168/jds.2010-3984

Brassard, L., Chaucheyras-Durand, F., Michalet-Doreau, B., & Martin, C. (2006). Dose effect of live yeasts on rumen microbial communities and fermentations during butyric latent acidosis in sheep: New type of interaction. *Animal Science*, *82*(6), 829–836. doi:10.1017/ASC200693

Bryant, M. P., & Small, N. (1956). The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *Journal of Bacteriology*, *82*, 16-21.

Chaucheyras-Durand, F., & Fonty, G. (2002). Influence of a probiotic yeast (*Saccharomyces cerevisiae* CNCM I-1077) on microbial colonization and fermentations in the rumen of newborn lambs. *Microbial Ecology in Health and Disease*, *14*(1), 30–36. doi: doi:10.1080/089106002760002739

Chaucheyras-Durand, F., Walker, N. D., & Bach, A. (2008). Effects of active dry yeasts on the rumen microbial ecosystem: Past, present and future. *Animal Feed Science and Technology*, *145*(1–4), 5–26. doi:10.1016/j.anifeedsci.2007.04.019

Chen, X., Xu, J., Shuai, J., Chen, J., Zhang, Z., & Fang, W. (2007). The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and Salmonella typhimurium. *International Journal of Food Microbiology*, *115*(3), 307–312. doi:10.1016/j.ijfoodmicro.2006.11.007

Chung, Y. H., Walker, N. D., McGinn, S. M., & Beauchemin, K. A. (2011). Differing effects of 2 active dried yeast (*Saccharomyces cerevisiae*) strains on ruminal acidosis and methane production in nonlactating dairy cows. *Journal of Dairy Science*, *94*(5), 2431–2439. doi:10.3168/jds.2010-3277

Clarke, R. T. J., Johns A. T., Coleman, G. S., Becker, E. R., & Usuelli, F. (1965). Role of the Rumen Ciliates in Bloat in Cattle. *Nature*, *205*(4966), 95–96.

Cochran, R. C., Adams, D. C., Wallace, J. D., & Galyean, M. L. (1986). Predicting digestibility of different diets with internal markers: Evaluation of four potential markers 1476-1483.

Czerkawski, J. W. (1986). *An introduction to rumen studies*. Oxfordshire: Pergamon Press.

De Koster, J. D., & Opsomer, G. (2013). Insulin resistance in dairy cows. *Veterinary Clinics of North America - Food Animal Practice 29*(2), 299-322. doi:10.1016/j.cvfa.2013.04.002

Dijkstra, J. (1994). Production and absorption of volatile fatty acids in the rumen. *Livestock Production Science, 39*, 61-69.

Dijkstra, J., Boer, H., van Bruchem, J., Bruining, M., & Tamminga, S. (1993). Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *British Journal of Nutrition*, *69*(2), 385–396. doi:10.1079/bjn19930041

Doreau, M., & Jouany, J. P. (1998). Effect of a *Saccharomyces cerevisiae* culture on nutrient digestion in lactating dairy cows. *Journal of Dairy Science*, *81*(12), 3214–3221. doi:10.3168/jds.S0022-0302(98)75885-0

Douglass, A. P., Offei, B., Braun-Galleani, S., Coughlan, A. Y., Martos, A. A. R., Ortiz-Merino, R. A., Byrne, K. P., & Wolfe, K. H. (2018). Population genomics shows no distinction between pathogenic *Candida krusei* and environmental *Pichia kudriavzevii*: One species, four names. *PLoS Pathogens*, *14*(7). doi: 10.1371/journal.ppat.1007138

Eadie, J. M. (1962). The development of rumen microbial populations in lambs and calves under various conditions of management. *J. Gen. Microbiol., 29*, 563-578.

Elahi, A., & Rehman, A. (2018). Bioconversion of hemicellulosic materials into ethanol by yeast, *Pichia kudriavzevii* 2-KLP1, isolated from industrial waste. *Revista Argentina de Microbiologia*, *50*(4), 417–425. doi:10.1016/j.ram.2017.07.008

Erickson, K. L., & Hubbard, N. E. (2000). Symposium: Probiotic bacteria: Implications for human health probiotic immunomodulation in health and disease. In *J. Nutr* (Vol. 130). https://academic.oup.com/jn/article/130/2/403S/4686432

Fernandes, T., Carvalho, B. F., Mantovani, H. C., Schwan, R. F., & Ávila, C. L. S. (2019). Identification and characterization of yeasts from bovine rumen for potential use as probiotics. *Journal of Applied Microbiology*, *127*(3), 845–855. doi:10.1111/jam.14350

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., Krehbiel, C. R., Nagaraja, T. G., Roe, B. A., & de Silva, U. (2010). Rumen microbial population dynamics during adaptation to a high-grain diet. *Applied and Environmental Microbiology*, *76*(22), 7482–7490. doi:10.1128/AEM.00388-10

Ferraretto, L. F., & Shaver, R. D. (2015). Effect of direct-fed microbial supplementation on lactation performance and total-tract starch digestibility by midlactation dairy cows. *Professional Animal Scientist*, *31*(1), 63–67. doi:10.15232/pas.2014-01369

France, J., & Dijkstra, J. (2005). Volatile Fatty Acid Production. In J. Dijkstra, J. M. Forbes, & J. France (Eds.), *Quantitative Aspects of Ruminant Digestion and Metabolism* (2nd ed., pp. 157–175). CABI Publishing.

Francisco, C. C., Chamberlain, C. S., Waldner, D. N., Wettemann, R. P., & Spicer, L. J. (2002). *Propionibacteria* fed to dairy cows: Effects on energy balance, plasma metabolites and hormones, and reproduction. *Journal of Dairy Science*, *85*(7), 1738–1751. doi:10.3168/jds.S0022-0302(02)74248-3

Franco, P. F., Ferreira, H. M., & Filho, E. X. F. (2004). Production and characterization of hemicellulase activities from *Trichoderma harzianum* strain T4. *Biotechnology and Applied Biochemistry*, *40*(3), 255. doi:10.1042/ba20030161

Franklin, S. T., Newman, M. C., Newman, K. E., & Meek, K. I. (2005). Immune parameters of dry cows fed mannan oligosaccharide and subsequent transfer of immunity to calves. *Journal of Dairy Science*, *88*(2), 766–775. doi:10.3168/jds.S0022-0302(05)72740-5

Fuller, R. (1989). A Review: Probiotics in man and animals. *Journal of Applied Bacteriology, 66*, 365-378.

Furman, O., Shenhav, L., Sasson, G., Kokou, F., Honig, H., Jacoby, S., Hertz, T., Cordero, O. X., Halperin, E., & Mizrahi, I. (2020). Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nature Communications*, *11*(1). doi:10.1038/s41467-020-15652-8

Gaffney, J., Embree, J., Gilmore, S., & Embree, M. (2021). *Ruminococcus bovis* sp. nov., a novel species of amylolytic *Ruminococcus* isolated from the rumen of a dairy cow. *International Journal of Systematic and Evolutionary Microbiology*, *71*(8). doi:10.1099/ijsem.0.004924

Ghorbani, G. R., Morgavi, D. P., Beauchemin, K. A., & Leedle, J. A. Z. (2002). Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. *J. Anim. Sci., 80,* 1977-1986. https://academic.oup.com/jas/article/80/7/1977/4789828

Goetz, B. M., Lefler, J., Abeyta, M. A., Horst, E. A., Mayorga, E. J., Al-Qaisi, M., Rodriguez-Jimenez, S., Martino, C., Izzo, A., La, R., Green, H. B., Moore, C. E., Embree, M., & Baumgard, L. H. (2021). Effects of dietary microbial feed supplement on production efficacy in lactating dairy cows. *Journal of Dairy Science Communications, 2,* 118-122.

Gonzalez-Recio, O., Zubiria, I., García-Rodríguez, A., Hurtado, A., & Atxaerandio, R. (2018). Short communication: Signs of host genetic regulation in the microbiome composition in 2 dairy breeds: Holstein and Brown Swiss. *Journal of Dairy Science, 101*(3), 2285–2292. doi:10.3168/jds.2017-13179

Grünberg, W., & Constable, P. D. (2009). Function and Dysfunction of the Ruminant Forestomach. In D. E. Anderson & D. M. Ring (Eds.), *Current veterinary therapy: Food animal practice.* (5th ed., pp. 12–19). Saunders Elsevier.

Gualdrón-Duarte, L. B., & Allen, M. S. (2018). Effects of acetic acid or sodium acetate infused into the rumen or abomasum on feeding behavior and metabolic response of cows in the postpartum period. *Journal of Dairy Science, 101*(3), 2016–2026. doi:10.3168/jds.2017-13609

Guzman, C. E., Bereza-Malcolm, L. T., de Groef, B., & Franks, A. E. (2015). Presence of selected methanogens, fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS ONE, 10*(7). doi:10.1371/journal.pone.0133048

Hall, M. B. (2009). Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. *Journal of AOAC International, 92*(1), 42-49

Haller, D., Colbus, H., Gänzle, M. G., Scherenbacher, P., Bode, C., & Hammes, W. P. (2001). Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: A comparative in vitro study between bacteria of intestinal and fermented food origin. *System. Appl. Microbiol, 24,* 218-226. http://www.urbanfischer.de/journals/sam

Hegarty, R. S., & Gerdes, R. (1999). Hydrogen production and transfer in the rumen. *Recent Advances in Animal Nutrition in Australia, 12.*

Heinrichs, A. J., Jones, C. M., & Heinrichs, B. S. (2003). Effects of mannan oligosaccharide or antibiotics in neonatal diets on health and growth of dairy calves. *Journal of Dairy Science*, *86*(12), 4064–4069. doi:10.3168/jds.S0022-0302(03)74018-1

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H., Abecia, L., Angarita, E., Aravena, P., Arenas, G. N., Ariza, C., Attwood, G. T., Avila, J. M., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M. F., Brown-Kav, A., … Zunino, P. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports*, *5*. doi:/10.1038/srep14567

Henry, W. A., & Morrison, F. B. (1928). *Feeds and feeding; a handbook for the student and stockman* (19th ed.). Henry-Morrison Co.

Hernández, J., Benedito, J. L., Abuelo, A., & Castillo, C. (2014). Ruminal acidosis in feedlot: From aetiology to prevention. *Scientific World Journal*, *2014*.

Hoang, V. T., Hoang, D. H., Pham, N. D., Tran, H. M., Bui, H. T. V., & Ngo, T. A. (2018). Hydrogen production by newly isolated *Clostridium* species from cow rumen in pure- and co-cultures on a broad range of carbon sources. *AIMS Energy*, *6*(5), 846–865. doi:10.3934/ENERGY.2018.5.846

Hossain, F. M. A., Islam, M. M., Ara, A., & Iliyas, N. (2014). Supplementing Probiotics (*Saccharomyces cerevisiae*) in multiparous crossbred cows ration provoke milk yield and composition. *Online Journal of Animal and Feed Research*, *4*(2), 18–24. http://www.science-line.com/in

Huang, Y., Marden, J. P., Julien, C., & Bayourthe, C. (2018). Redox potential: An intrinsic parameter of the rumen environment. *Journal of Animal Physiology and Animal Nutrition*, *102*(2), 393-402. doi:10.1111/jpn.12855

Hue, L., & Taegtmeyer, H. (2009). The Randle cycle revisited: a new head for an old hat. *Am. J. Physiol. Endocrinol. Metab.*, *297*, 578–591. doi:10.1152/ajpendo.00093.2009.-In

Huntington, G. B. (1989). Energy metabolism in the digestive tract and liver of cattle: influence of physiological state and nutrition. *Reproduction Nutrition Development*, *30*(1), 35-47.

Ibuki, M., Fukui, K., Kanatani, H., & Mine, Y. (2014). Anti-inflammatory effects of mannanase-hydrolyzed copra meal in a porcine model of colitis. *Journal of Veterinary Medical Science*, *76*(5), 645–651. doi:10.1292/jvms.13-0424

Intanoo, M., Kongkeitkajorn, M. B., Suriyasathaporn, W., Phasuk, Y., Bernard, J. K., & Pattarajinda, V. (2020). Effect of supplemental *Kluyveromyces marxianus* and *Pichia kudriavzevii* on aflatoxin M1 excretion in milk of lactating dairy cows. *Animals*, *10*(4). doi:10.3390/ani10040709

Isolauri, E., Sütas, Y., Kankaanpää, H. A., & Salminen, S. (2001). Probiotics: effects on immunity. *The American Journal of Clinical Nutrition*, *73*(2), 444–450.

Izumi, K., Fukumori, R., Oikawa, S., & Oba, M. (2019). Short communication: Effects of butyrate supplementation on the productivity of lactating dairy cows fed diets differing in starch content. *Journal of Dairy Science*, *102*(12), 11051–11056. doi:10.3168/jds.2019-17113

Joblin, K. N. (1981). Isolation, Enumeration, and Maintenance of Rumen Anaerobic Fungi in Roll Tubes. *Applied and Environmental Microbiology*, 1119-1122.

Jones, G. W., & Rutter, J. M. (1972). Role of the K88 Antigen in the Pathogenesis of Neonatal Diarrhea Caused by *Escherichia coli* in Piglets. *Infection and Immunity*, *6*(6), 918-927.

Jouany, J. P. (2006). Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Animal Reproduction Science*, *96*(3–4), 250–264. doi:10.1016/j.anireprosci.2006.08.005

Julien, C., Marden, J.-P., Auclair, E., Cauquil, L., & Bayourthe, C. (2020). Reducing conditions varied with diets and bacterial communities in the rumen on dairy cows. *Gut Microbiology, 7. Joint Symposium*. https://hal.inrae.fr/hal-02817627

Jurgens, M. N. (2002). *Animal Feeding and Nutrition* (9th ed.). Kendall/Hunt.

Kenney, N. (2013). Impact of direct-fed microbials on nutrient utilization in beef impact of direct-fed microbials on nutrient utilization in beef cattle. *Thesis and Dissertations – Animal and Food Sciences.* 17. https://uknowledge.uky.edu/animalsci_etds/17

Kepler, C. R., Hirons, K. P., McNeill, J. J., & Tove, S. B. (1966). Intermediates and products of the biohydrogenation of linoleic acid by *Butyrinvibrio fibrisolvens*. *Journal of Biological Chemistry*, *241*(6), 1350–1354. doi:10.1016/s0021-9258(18)96781-5

Klieve, A. v., McLennan, S. R., & Ouwerkerk, D. (2012). Persistence of orally administered *Megasphaera elsdenii* and *Ruminococcus bromii* in the rumen of beef cattle fed a high grain (barley) diet. *Animal Production Science*, *52*(5), 297–304. doi:10.1071/AN11111

Kritchevsky, D. (2000). Antimutagenic and some other effects of conjugated linoleic acid. *British Journal of Nutrition, 83*(5), 459-465. CAB International. doi:10.1017/s0007114500000581

Křížová, L., Richter, M., Třináctý, J., Říha, J., & Kumprechtová, D. (2011). The effect of feeding live yeast cultures on ruminal pH and redox potential in dry cows as continuously measured by a new wireless device. *Czech J. Anim. Sci., 56*(1), 37-45.

Kung, L., & Hession, A. 0. (1995). Preventing in vitro lactate accumulation in ruminal fermentations by inoculation with *Megasphaera elsdenii*. *Journal of Animal Science, 73,* 250-256. https://academic.oup.com/jas/article/73/1/250/4564214

Kurtzman, C. P., Fell, J. W., & Boekhout, T. (Eds.). (2011). *The Yeasts, a Taxonomic Study* (5th ed., Vol. 1). Elsevier.

Lawrence, M., Polukis, S., Barnard, A., Miller, M., Kung, L., Gressley, T. (2021). Evaluating the effects of *Lactobacillus animalis* and *Propionibacterium freudenreichii* on performance and rumen and fecal measures in lactating dairy cows. *Journal of Dairy Science, 104*(4), 4119-4133. Doi: https://doi.org/10.3168/jds.2020-19291

Lee, S. S., Hsu, J.-T., Mantovani, H. C., & Russell, J. B. (2002). The effect of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, on ruminal methane production in vitro. *FEMS Microbiology Letters, 217*, 51-55. www.fems-microbiology.org

Leedle, J. A. Z., Bryant, M. P., & Hespell, R. B. (1982). Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low-or high-forage diets. *Applied and Environmental Microbiology, 44*(2), 402-412. https://journals.asm.org/journal/aem

Lefler, J., Minini, S., Morandi, A., & Embree, M. (2020). Effect of direct-fed microbial on commercial dairy dry-lot (Abstract). In *Journal of Dairy Science* (Vol. 103, Issue Suppl. 1, p. S203).

Leschine, S. (2005). Degradation of polymers: Cellulose, xylan, pectin, starch. In P. Dürre (Ed.), *Handbook on Clostridia* (pp. 101–121). Taylor & Francis.

Lima, J. R., Ribon, A. D. O. B., Russell, J. B., & Mantovani, H. C. (2009). Bovicin HC5 inhibits wasteful amino acid degradation by mixed ruminal bacteria in vitro. *FEMS Microbiology Letters*, *292*(1), 78–84. doi:10.1111/j.1574-6968.2008.01474.x

Liu, E., Hanigan, M., VandeHaar, M. (2021). Importance of considering body weight change in response to dietary protein deficiency in lactating dairy cows. *Journal of Dairy Science, 104*(11), 11567-11579. Doi:10.3168/jds.2020-19566

Lorenz, I. (2016). Subacute ruminal acidosis. In *The Merck veterinary manual* (11th ed.). Merck & Co., Inc.

Maamouri, O., & Ben Salem, M. (2021). The effect of live yeast *Saccharomyces cerevisiae* as probiotic supply on growth performance, feed intake, ruminal pH and fermentation in fattening calves. *Veterinary Medicine and Science*. doi:10.1002/vms3.631

Magnoli, A. P., Rodriguez, M. C., González Pereyra, M. L., Poloni, V. L., Peralta, M. F., Nilson, A. J., Miazzo, R. D., Bagnis, G., Chiacchiera, S. M., & Cavaglieri, L. R. (2017). Use of yeast (*Pichia kudriavzevii*) as a novel feed additive to ameliorate the effects of aflatoxin B1 on broiler chicken performance. *Mycotoxin Research, 33*(4), 273–283. doi:10.1007/s12550-017-0285-y

Malmuthuge, N., Griebel, P. J., & Guan, L. L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. *Applied and Environmental Microbiology, 80*(6), 2021–2028. doi:10.1128/AEM.03864-13

Mantovani, H. C., Russell, J. B., Rio, H., & Mantovani, C. (2002). The bacteriocins of ruminal bacteria and their potential as an alternative to antibiotics. *Journal of Molecular Microbiology and Biotechnology, 4*(4), 347-355. www.caister.com/bacteria-plant

Mathieu, F., Jouany, J. P., Sénaud, J., Bohatier, J., Bertin, G., & Mercier, M. (1996). The effect of *Saccharomyces cerevisiae* and *Aspergillus oryzae* on fermentations in the rumen of faunated and defaunated sheep; protozoal and probiotic interactions. *Reproduction Nutrition Development, 36*(3), 271-287.

McAllister, T. A., Beauchemin, K. A., Alazzeh, A. Y., Baah, J., Teather, R. M., & Stanford, K. (2011). Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. *Canadian Journal of Animal Science, 91*(2), 193-211. doi:10.4141/cjas10047

McArt, J. A. A., Nydam, D. v., Oetzel, G. R., Overton, T. R., & Ospina, P. A. (2013). Elevated non-esterified fatty acids and β-hydroxybutyrate and their association with transition dairy cow performance. *Veterinary Journal, 198*(3), 560-570. doi:10.1016/j.tvjl.2013.08.011

McCann, J. C., Luan, S., Cardoso, F. C., Derakhshani, H., Khafipour, E., & Loor, J. J. (2016). Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. *Frontiers in Microbiology, 7*(MAY). doi:10.3389/fmicb.2016.00701

Mendoza, G. D., Britton, R. A., & Stock, R. A. (1993). Influence of ruminal protozoa on site and extent of starch digestion and ruminal fermentation. *Journal of Animal Science, 71*, 1572-1578. https://academic.oup.com/jas/article/71/6/1572/4718931

Miettinen, M., Vuopio-Varkila, J., & Varkila, K. (1996). Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infection and Immunity, 64*(12), 5403-5405.

Moraïs, S., & Mizrahi, I. (2019). The Road Not Taken: The Rumen Microbiome, Functional Groups, and Community States. *Trends in Microbiology, 27*(6), 538-549. Elsevier Ltd. doi:10.1016/j.tim.2018.12.011

Moran, J. (2005). How the Rumen Works. In *Tropical Dairy Farming: Feeding Management for Small Holder Dairy Farmers in the Humid Tropics* (pp. 43–49). CSIRO Publishing.

Mrázek, J., Tepšiý, K., Avguštin, G., Kopeýný, J., & Marinšek-Logar, R. (2006). Diet-dependent shifts in ruminal butyrate-producing bacteria. *Folia Microbiol, 51*(4), 294-298. http://www.biomed.cas.cz/mbu/folia/

Mutsvangwa, T., Walton, J. P., Plaizier, J. C., Duffield, T. F., Bagg, R., Dick, P., Vessie, G., & McBride, B. W. (2002). Effects of a monensin controlled-release capsule or premix on attenuation of subacute ruminal acidosis in dairy cows. *Journal of Dairy Science, 85*(12), 3454–3461. doi:10.3168/jds.S0022-0302(02)74433-0

Nagaraja, T. G., & Titgemeyer, E. C. (2007). Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. *Journal of Dairy Science, 90*(S), E17–E38. doi:10.3168/jds.2006-478

Nakai, Y., Nakatani, M., Ando, T., Inamoto, T., & Ogimoto, K. (1993). *Degradation of β-mannan by rumen bacteria*.

Natalicia Mendes de Almeida, P., Robson Duarte, E., Oliveira Abrão, F., Eduardo Silva Freitas, C., Castro Geraseev, L., & Augusto Rosa, C. (2012). Aerobic fungi in the rumen fluid from dairy cattle fed different sources of forage. *Revista Brasileira de Zootecnia, 41*(11), 2336-2342. doi:www.sbz.org.br

NASEM. (2021). Nutrient requirements of dairy cattle: Eight revised edition. Washington, DC: The National Academies Press. Doi: 10.17226/25806

Newbold, C. J., Wallace, R. J., & Mcintosh, F. M. (1996). Mode of action of the yeast *Saccharomyces cerevisiae* as a feed additive for ruminants. *British Journal of Nutrition, 76*(2), 249–261. doi:10.1079/bjn19960029

Nocek, J. E., & Kautz, W. P. (2006). Direct-fed microbial supplementation on ruminal digestion, health, and performance of pre- and postpartum dairy cattle. *Journal of Dairy Science*, *89*(1), 260–266. doi:10.3168/jds.S0022-0302(06)72090-2

Nocek, J. E., Kautz, W. P., Leedle, J. A. Z., & Allman, J. G. (2002). Ruminal supplementation of direct-fed microbials on diurnal pH variation and in situ digestion in dairy cattle. *Journal of Dairy Science*, *85*(2), 429–433. doi:10.3168/jds.S0022-0302(02)74091-5

Nocek, J. E., Kautz, W. P., Leedle, J. A. Z., & Block, E. (2003). Direct-fed microbial supplementation on the performance of dairy cattle during the transition period. *Journal of Dairy Science*, *86*(1), 331–335. doi:10.3168/jds.S0022-0302(03)73610-8

Oviedo-Boyso, J., Valdez-Alarcón, J. J., Cajero-Juárez, M., Ochoa-Zarzosa, A., López-Meza, J. E., Bravo-Patiño, A., & Baizabal-Aguirre, V. M. (2007). Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *Journal of Infection*, *54*(4), 399–409. doi:10.1016/j.jinf.2006.06.010

Philippeau, C., Lettat, A., Martin, C., Silberberg, M., Morgavi, D. P., Ferlay, A., Berger, C., & Nozière, P. (2017). Effects of bacterial direct-fed microbials on ruminal characteristics, methane emission, and milk fatty acid composition in cows fed high- or low-starch diets. *Journal of Dairy Science*, *100*(4), 2637–2650. doi:10.3168/jds.2016-11663

Polan, C. E., McNeill, J. J., & Tove, S. B. (1964). Biohydration of unsaturated fatty acids by the rumen bacteria. *Journal of Bacteriology, 88*(4), 1056-1064.

Qvirist, L., Vorontsov, E., Veide Vilg, J., & Andlid, T. (2017). Strain improvement of *Pichia kudriavzevii* TY13 for raised phytase production and reduced phosphate repression. *Microbial Biotechnology*, *10*(2), 341–353. doi:10.1111/1751-7915.12427

Raeth-Knight, M. L., Linn, J. G., & Jung, H. G. (2007). Effect of direct-fed microbials on performance, diet digestibility, and rumen characteristics of Holstein dairy cows. *Journal of Dairy Science*, *90*(4), 1802–1809. doi:10.3168/jds.2006-643

Rainard, P., Cunha, P., & Gilbert, F. B. (2016). Innate and adaptive immunity synergize to trigger inflammation in the mammary gland. *PLoS ONE*, *11*(4). doi:10.1371/journal.pone.0154172

Rainard, P., Cunha, P., Ledresseur, M., Staub, C., Touzé, J. L., Kempf, F., Gilbert, F. B., & Foucras, G. (2015). Antigen-specific mammary inflammation depends on the production of IL-17A and IFN-γ by bovine CD4+ T lymphocytes. *PLoS ONE*, *10*(9). doi:10.1371/journal.pone.0137755

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., & Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *Journal of Applied Microbiology, 116*(2), 245–257. doi:10.1111/jam.12405

Riley, M. A., & Wertz, J. E. (2002). Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie, 84*, 357-364

Roche, J. R., Friggens, N. C., Kay, J. K., Fisher, M. W., Stafford, K. J., & Berry, D. P. (2009). Body condition score and its association with dairy cow productivity, health, and welfare. *Journal of Dairy Science, 92*(12), 5769-5801 doi:10.3168/jds.2009-2431

Rossow, H. A., Riordan, T., & Riordan, A. (2018). Effects of addition of a live yeast product on dairy cattle performance. *Journal of Applied Animal Research, 46*(1), 159–163. doi:10.1080/09712119.2017.1281810

Santos, M. C., Lock, A. L., Mechor, G. D., & Kung, L. (2015). Effects of a spoilage yeast from silage on in vitro ruminal fermentation. *Journal of Dairy Science*, *98*(4), 2603–2610. doi:10.3168/jds.2014-8683

Schoch, C. L., & et al. (2020). *NCBI Taxonomy Browser*.

Sechovcová, H., Kulhavá, L., Fliegerová, K., Trundová, M., Morais, D., Mrázek, J., & Kopečný, J. (2019). Comparison of enzymatic activities and proteomic profiles of *Butyrivibrio fibrisolvens* grown on different carbon sources. *Proteome Science, 17*(1). doi:10.1186/s12953-019-0150-3

Seo, J. K., Kim, S.-W., Kim, M. H., Upadhaya, S. D., Kam, D. K., & Ha, J. K. (2010). Direct-fed microbials for ruminant animals. *Asian-Australasian Journal of Animal Sciences*, *23*(12), 1657–1667.

Sheperd, A. C., & Combs, D. K. (1998). Long-term effects of acetate and propionate on voluntary feed intake by midlactation cows. *Journal of Dairy Science, 81*(8), 2240–2250. doi:10.3168/jds.S0022-0302(98)75803-5

Sherman, P. M., Johnson-Henry, K. C., Yeung, H. P., Ngo, P. S. C., Goulet, J., & Tompkins, T. A. (2005). Probiotics reduce enterohemorrhagic *Escherichia coli* O157:H7- and enteropathogenic *E. coli* O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. *Infection and Immunity*, *73*(8), 5183–5188. doi:10.1128/IAI.73.8.5183-5188.2005

Simkins, K. L., Suttie, J. W., & Baumgardt, B. R. (1965). Regulation of food intake in ruminants. 4. Effect of acetate, propionate, butyrate, and glucose on voluntary food intake in dairy cattle. *Journal of Dairy Science*, *48*(12), 1635–1642. doi:10.3168/jds.S0022-0302(65)88541-1

Stein, D. R., Allen, D. T., Perry, E. B., Bruner, J. C., Gates, K. W., Rehberger, T. G., Mertz, K., Jones, D., & Spicer, L. J. (2006). Effects of feeding *Propionibacteria* to dairy cows on milk yield, milk components, and reproduction. *Journal of Dairy Science*, *89*(1), 111–125. doi:10.3168/jds.S0022-0302(06)72074-4

Stella, A. v., Paratte, R., Valnegri, L., Cigalino, G., Soncini, G., Chevaux, E., Dell'Orto, V., & Savoini, G. (2007). Effect of administration of live *Saccharomyces cerevisiae* on milk production, milk composition, blood metabolites, and faecal flora in early lactating dairy goats. *Small Ruminant Research*, *67*(1), 7–13. doi:10.1016/j.smallrumres.2005.08.024

Stevens, C. E. (1970). Fatty acid transport through the rumen epithelium. *Physiology of Digestion and Metabolism in the Ruminant; Proceedings of the Third International Symposium*, 101–112.

Stewart, C. S. (1977). Factors Affecting the Cellulolytic Activity of Rumen Contents. *Applied and Environmental Microbiology*, *33*(3), 497-502 https://journals.asm.org/journal/aem

Stewart, C. S., Flint, H. J., & Bryant, M. P. (1997). *The Rumen Microbial Ecosystem* (P. N. Hobson & C. S. Steward, Eds.; Second Edition). Blackie Academic & Professional.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R., & Watson, M. (2019). Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nature Biotechnology*, *37*(8), 953–961. doi:10.1038/s41587-019-0202-3

Stock, R., & Britton, R. (1991). *Acidosis*. https://digitalcommons.unl.edu/extensionhist/198

Storm, E., Brown, D. S., & Ørskov, E. R. (1983). The nutritive value of rumen micro-organisms in ruminants. *British Journal of Nutrition*, *50*(2), 479–485. doi:10.1079/bjn19830116

Suntara, C., Cherdthong, A., Uriyapongson, S., Wanapat, M., & Chanjula, P. (2021). Novel Crabtree negative yeast from rumen fluids can improve rumen fermentation and milk quality. *Scientific Reports*, *11*(1). doi:10.1038/s41598-021-85643-2

Tas, M. v., Evans, R. A., & Axford, R. F. E. (1981). The digestibility of amino acids in the small intestine of the sheep. *British Journal of Nutrition*, *45*(1), 167–174. doi:10.1079/bjn19810089

Tejada-Simon, M. V., & Pestka, J. J. (1999). Proinflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. *Journal of Food Protection, 62*(12), 1435-1444. http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf

Tesfaye, A., & Hailu, Y. (2019). The effects of probiotics supplementation on milk yield and composition of lactating dairy cows. *The Journal of Phytopharmacology, 8*(1), 12-17. www.phytopharmajournal.com

Tewoldebrhan, T. A., Appuhamy, J. A. D. R. N., Lee, J. J., Niu, M., Seo, S., Jeong, S., & Kebreab, E. (2017). Exogenous β-mannanase improves feed conversion efficiency and reduces somatic cell count in dairy cattle. *Journal of Dairy Science*, *100*(1), 244–252. doi:10.3168/jds.2016-11017

The United States Food and Drug Administration. (1995). *FDA CPG Sec. 689.100 Direct-Fed Microbial Products*.

Thrune, M., Bach, A., Ruiz-Moreno, M., Stern, M. D., & Linn, J. G. (2009). Effects of Saccharomyces cerevisiae on ruminal pH and microbial fermentation in dairy cows. Yeast supplementation on rumen fermentation. *Livestock Science*, *124*(1–3), 261–265. doi:10.1016/j.livsci.2009.02.007

Torres, C. (2021, November 11). Tiny microbes power dairy feed additive. *FarmProgress*.

Urrutia, N., Bomberger, R., Matamoros, C., & Harvatine, K. J. (2019). Effect of dietary supplementation of sodium acetate and calcium butyrate on milk fat synthesis in lactating dairy cows. *Journal of Dairy Science*, *102*(6), 5172–5181. doi:10.3168/jds.2018-16024

van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, *74*(10), 3583–3597. doi:10.3168/jds.S0022-0302(91)78551-2

Vijayakumar, M., Park, J., Ki, K., Lim, D., Kim, S., Park, S., Jeong, H., Park, B., and Kim, T. (2017). The effect of lactation number, stage, length, and milking frequency on milk yield in Korean Holstein dairy cows using automatic milking system. *Asian-Australasian Journal of Animal Science, 30*(8), 1093-1098. doi: 10.5713/ajas.16.0882

Wallace, R. J., Sasson, G., Garnsworthy, P. C., Tapio, I., Gregson, E., Bani, P., Huhtanen, P., Bayat, A. R., Strozzi, F., Biscarini, F., Snelling, T. J., Saunders, N., Potterton, S. L., Craigon, J., Minuti, A., Trevisi, E., Callegari, M. L., Piccioli Cappelli, F., Cabezas-Garcia, E. H., … Mizrahi, I. (2019). A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. *Sci. Adv.*, *5*, 8391-8394. http://advances.sciencemag.org/

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. *Frontiers in Microbiology*, *6*. Frontiers Media S.A. doi:10.3389/fmicb.2015.00296

Weimer, P. J., Stevenson, D. M., Mantovani, H. C., & Man, S. L. C. (2010). Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents1. *Journal of Dairy Science*, *93*(12), 5902–5912. doi:10.3168/jds.2010-3500

Weiss, W. P., Wyatt, D. J., & McKelvey, T. R. (2008). Effect of feeding P*ropionibacteria* on milk production by early lactation dairy cows. *Journal of Dairy Science*, *91*(2), 646–652. doi:10.3168/jds.2007-0693

West, J. W., & Bernard, J. K. (2011). Effects of addition of bacterial inoculants to the diets of lactating dairy cows on feed intake, milk yield, and milk composition. *Professional Animal Scientist*, *27*(2), 122–126. doi:10.15232/S1080-7446(15)30458-7

Wildman, E. E., Jones, G. M., Wagner, P. E., Boman, R. L., Troutt, H. F., & Lesch, T. N. (1982). A dairy cow body condition scoring system and its relationship to selected production characteristics. *Journal of Dairy Science*, *65*(3), 495–501. doi:10.3168/jds.S0022-0302(82)82223-6

Williams, A. G., & Coleman, G. S. (1997). The rumen protozoa. In P. N. Hobson & C. S. Stewart (Eds.), *The Rumen Microbial Ecosystem* (2nd ed., pp. 73–139). Blackie Academic and Professional.

Xia, Y., Kong, Y., Seviour, R., Yang, H. E., Forster, R., Vasanthan, T., & McAllister, T. (2015). In situ identification and quantification of starch-hydrolyzing bacteria attached to barley and corn grain in the rumen of cows fed barley-based diets. *FEMS Microbiology Ecology*, *91*(8). doi:10.1093/femsec/fiv077

Xu, H., Huang, W., Hou, Q., Kwok, L. yu, Sun, Z., Ma, H., Zhao, F., Lee, Y. K., & Zhang, H. (2017). The effects of probiotics administration on the milk production, milk components and fecal bacteria microbiota of dairy cows. *Science Bulletin*, *62*(11), 767–774. doi:10.1016/j.scib.2017.04.019

Yáñez-Ruiz, D. R., Abecia, L., & Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: A review. *Frontiers in Microbiology, 6.* Frontiers Media S.A. doi:10.3389/fmicb.2015.01133

Yoon, I. K., & Stern, M. D. (1995). Influence of direct-fed microbials on ruminal microbial fermentation and performance of ruminants: A review. *Asian-Australasian Journal of Animal Sciences, 8*(6), 533–555.

Yoon, I. K., & Stern, M. D. (1996). Effects of *Saccharomyces cerevisiae* and *Aspergillus oryzae* cultures on ruminal fermentation in dairy cows. *Journal of Dairy Science, 79*(3), 411–417. doi:10.3168/jds.S0022-0302(96)76380-4

Zimbelman, R., Rhoads, R., Rhoads, M., Duff, G., Baumgard, L., Collier, R. (2009). A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. *Southwest Nutrition & Management Conference,* 158-168.

Zhou, M., Peng, Y. J., Chen, Y., Klinger, C. M., Oba, M., Liu, J. X., & Guan, L. L. (2018). Assessment of microbiome changes after rumen transfaunation: implications on improving feed efficiency in beef cattle. *Microbiome*, *6*(1), 62. doi:10.1186/s40168-018-0447-y

Ziolecki, B. A., & E Briggs, C. A. (1961). The microflora of the rumen of the young calf: II. Source, nature, and development. *J. appl. Bact.*, *24*(2), 148-163.