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In-Vivo Assessment of a Direct-Fed Microbial on Lactation Performances, Blood Biomarkers, Ruminal Fermentation and Microbial Abundance in Transition to Mid-Lactation Holstein Cows

Marcela Bulnes Lopez
South Dakota State University, Marcela.BulnesLopez@sdstate.edu

IN-VIVO ASSESSMENT OF A DIRECT-FED MICROBIAL ON LACTATION PERFORMANCES, BLOOD BIOMARKERS, RUMINAL FERMENTATION AND MICROBIAL ABUNDANCE IN TRANSITION TO MID-LACTATION HOLSTEIN COWS.

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MARCELA BULNES LOPEZ

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Md Elias Uddin
Advisor Date

Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

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LIST OF ABBREVIATIONS

BCS	Body condition score
BHB	B-hydroxybutyrate
BW	Body weight
CON	Control
d	Days
DFM	Direct-fed microbial
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EB	Energy balance
ECM	Energy-corrected milk
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NH3	Ammonia
SCC	Somatic count cell
TMR	Total mix ratio
VFA	Volatile fatty acids

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ABSTRACT

IN-VIVO ASSESSMENT OF DIRECT-FED MICROBIAL ON LACTATION PERFORMANCES, BLOOD BIOMARKERS, RUMINAL FERMENTATION AND MICROBIAL ABUNDANCE IN HOLSTEIN COWS.

MARCELA BULNES LOPEZ

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The transition period is a crucial stage in the lactation cycle and can lead to significant metabolic changes in cows. The use of nutritional interventions, such as direct-fed microbials, may assist cows during the transition from pregnancy to early lactation, reducing the occurrence of metabolic disorders and improving overall health. The primary goal of this study was to assess the effects of a commercial rumen-derived direct-fed microbial (DFM) product (Galaxis™ Frontier (GF) Native Microbials Inc., CA, USA) on various factors such as performance, blood biomarkers, rumen fermentation, and bacterial population in dairy cows during the transition period up to 100 d in milk (DIM). Overall, the results showed that rumen derived DFM supplementation could promote positive responses on performance, such as milk yield and feed efficiency. In the rumen, the DFM product contributed to increased butyrate and valerate, accompanied by an improved ruminal abundance of lactic acid-utilizing bacteria. Moreover, DFM supplementation may have influenced lipid metabolism, leading to greater oxidative stress and inflammation within non-pathological levels. In conclusion, our study suggests that supplementing DFM during the transition period to mid-lactation can positively

impact lactation performance and the rumen environment, indicating an overall beneficial effect.

INTRODUCTION

Ruminants, such as dairy cows, have a specialized digestive system to ferment ingested feed by microbial organisms (Niwiska, 2012). The rumen is the first and largest compartment of the four-compartment stomach, and it is here where microbial fermentation mainly occurs. The fermentation of feed by the microbes in the rumen produces volatile fatty acids (VFA) (McDonald et al., 2011). In order to ensure normal microbial growth in the rumen, it is essential to maintain the ecological conditions (e.g., pH, temperature, anaerobic environment) regulating the microbial population.

Although countless microbes are found in the ruminant's digestive tract, only the microbiota of the rumen has a true symbiotic relationship with the host (Church, 1988). The rumen microorganisms are either anaerobic or facultatively anaerobic and can be divided into three main groups: bacteria, protozoa and fungi (Czerkawski, 1986). Rumen microbial community structure is affected by several factors such as diet, geographical location, breed or age of animals (Tapio et al., 2017).

The period spanning from three weeks prior to and three weeks following parturition is commonly referred to as the transition period (Drackley, 1999). During this period, cows are transitioning from high-forage to high-grain diets. Additionally, the transition period is linked to the highest occurrence of issues with production, metabolic disorders, and infectious diseases in dairy cows. During the transition period, cows experience a negative energy balance due to increased energy needs, leading to elevated levels of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) in the

bloodstream, which can result in metabolic disorders. The cow's immune system is also suppressed during this period, potentially affecting immune function.

The progress in transition cow research has paved the way for the formulation of nutritional approaches to support cows during the transition from pregnancy to early lactation. Direct-fed microbials (DFM) are feed additives commonly supplemented to improve production, efficiency and health in dairy cows (Goldsmith et al., 2023). Several studies have reported the positive effects of supplementing DFM on the performance of dairy cows during the transition period, as well as in early-and mid-lactation (Nocek & Kautz, 2006; Kumprechtová et al., 2019; Oh et al., 2019; Hiltz et al., 2023).

Our general hypothesis is that supplementing a rumen-derived DFM during the transition period through 100 DIM will influence lactation performances and rumen environment, and consequently improving health and performance in transition to mid-lactation dairy cows. The objective of this study was to evaluate the effects of a rumen-derived DFM [Galaxis™ Frontier (GF); Native Microbials, Inc., CA, USA] on performances, blood biomarkers, rumen fermentation profile, and bacterial abundance in dairy cows from transition period until 100 DIM.

CHAPTER 1. LITERATURE REVIEW

1. The ruminant digestive system

Unlike monogastric animals, the anatomical and physiological adaptation of the ruminant digestive system has allowed them to utilize forages and fibrous roughages containing cellulose and other resistant carbohydrates as food sources (Van Soest, 1994c). This specialized digestive system involves fermenta6tion of the ingested feed by microbial organisms before it reaches gastric and intestinal digestion activity; this distinct feature allows them to extract nutrients from fibrous plant sources that would otherwise be unavailable (Niwiska, 2012). The ruminant's digestive anatomy is characterized by a multichambered stomach composed of the reticulum, rumen, omasum, and abomasum (Figure 1.1). The first two compartments are often called the reticulorumen (McDonald et al., 2011). The food enters through the esophagus into the reticulorumen, then follows to the omasum. The latter contains many internal folds, and then is directed toward the abomasum, the acid-secreting stomach (Czerkawski, 1986). These different compartments work together in a synchronized way to provide an ideal environment for fermentation, blend ingested feed with microorganisms, reduce the particle size of feeds, remove fermentation gases, and control the rate of passage of digesta to the lower gut (Russell, 2002). The rumen, the largest compartment, is the first chamber of the four-compartment stomach, and it is here where microbial fermentation mainly occurs. The fermentation of feed by the microbes in the rumen produces volatile fatty acids (VFA) (McDonald et al., 2011).

1.1 Rumen environment

The rumen is a continuous culture system often described as a biological fermentation unit (Ruckebusch & Thivend, 1980; McDonald et al., 2011). In order to ensure normal microbial growth and metabolism in the rumen, it is essential to maintain the ecological conditions that act as regulators of the microbial population. This includes factors such as pH, temperature, and the availability of nutrients. The rumen provides a moist, warm, buffered, anaerobic, rich in substrate environment ideal for the growth of anaerobic bacteria, protozoa and fungi. The rumen functions as a continuous culture system due to the constant supply of substrate, removal of end products (e.g. VFAs, CH_4, CO_2, ammonia-N and microbial cells) , and removal of undigested feed and waste products (Czerkawski, 1986; Church, 1988; McDonald et al., 2011).

The rumen requires several homeostatic mechanisms to maintain an optimal pH. The latter ranges from 5.5 to 7.2, with lower pH values detected shortly after high concentrate meals (Church, 1988). The saliva contains phosphate and bicarbonate that act as pH buffers. In addition, the rapid absorption of VFA by the rumen helps stabilize the pH (McDonald et al., 2011). When the ruminal pH falls below 5.5, high concentrations of total VFA or lactic acid can be accumulated in the rumen, leading to subacute or acute rumen acidosis (Ban & Guan, 2021). The anaerobic condition in the rumen is indicated by a negative oxidation-reduction potential (E_h) between -250 to 450 millivolts (Church, 1988). In order to maintain an anaerobic condition for the microbes, oxygen entering with the feed is rapidly consumed by facultative anaerobes and yeast. In the absence of oxygen, carbon is the ultimate acceptor of hydrogen ions, which allows methane

formation (McDonald et al., 2011). Additionally, the temperature of the rumen remains relatively constant at 39°C (Russell & Hespell, 1981).

Rumen contents normally contain 850 to 930 g water/kg (McDonald et al., 2011). These aqueous conditions provide ideal microbial interactions and high activities of microbial enzymes (Church, 1988). Rumen contents are stratified in several layers with specific gravity being the major separation factor (Welch, 1986). The top layer consists of large fibers (>1 cm) and contains less fluid content. The middle layer contains fibers fragmented though rumination into smaller pieces below approximately 1cm with an increased fluid to fiber ratio. Finally, the bottom layer consists primarily of rumen fluid and fine fiber particles.

1.2 Rumen function

The contents in the rumen are continuously mixed by rhythmic contractions (McDonald et al., 2011). Rumination is an important cyclic process part of the ruminant physiology, and it is characterized by regurgitation, remastication, and reswallowing (Church, 1988; Van Soest, 1994a; Simoni et al., 2023). When the ingested feed is too large or coarse, it will be broken down into fine particles through rumination. During rumination, the ingesta from the reticulum is drawn back to the esophagus and into the mouth (regurgitation), where it is remasticated and reswallowed (Van Soest, 1994a). The primary reason stimulating rumination is likely the tactile stimulation of the anterior rumen epithelium, hence diet plays an important role in stimulation for rumination (McDonald et al., 2011). Increased amounts of fiber in the diet stimulate rumination and reduce VFA production. In contrast, reducing fiber in the diet will decrease rumination, leading to less salivary buffer secretion, and decrease in rumen pH reflected in altered

rumen fermentation (Mertens, 1996). Moreover, rumination time and interval time between rumination can be used to predict dry matter intake (Clement et al., 2014).

The feed ingested by the ruminant is retained in the rumen and undergoes microbial digestion and fermentation (Hungate, 1975; Niwiska, 2012). The purpose of fermentative digestion in the rumen is to convert ingested feedstuff into microbial cells and end products (particularly VFAs). These products serve as a source of energy and protein for the animal (Russell & Hespell, 1981). During ruminal fermentation, carbohydrates and proteins are degraded into short-term intermediates such as sugars and amino acids. Microbes then further metabolize these intermediates to produce various byproducts of rumen fermentation. From the host standpoint, these can be useful (VFAs, microbial protein, B-vitamins), less useful (carbon dioxide, methane) or even harmful (ammonia, nitrate) (Church, 1988; Niwiska, 2012). The relative proportions of VFAs produced during ruminal fermentation vary, with acetic acid being the most abundant, followed by propionic, butyric, iso-butyric, valeric, and iso-valeric acids in descending order of usual abundance. These proportions can be significantly affected by the animal's diet and the composition of the microbial population in the rumen, particularly the methanogens (Van Soest, 1994a; Krehbiel, 2014). Strategies to manipulate rumen fermentation processes to optimize rumen performance should benefit both microbes and the host. The VFA produced are not utilized for energy by microbes but are readily absorbed and utilized as energy for host metabolism, making this relationship truly symbiotic (Pitta et al., 2018).

2. Rumen microbiome

Although a countless number of microbes are found in the digestive tract of the ruminant, only the microbiota of the rumen have a truly symbiotic relationship with the host (Church, 1988). Ruminants lack the enzymes required to break down complex plant polysaccharides. They rely on the microbes present in their rumen to digest the ingested plant material (Van Soest, 1994b; Henderson et al., 2015). The rumen microbiome is a diverse and complex array of microbial groups that perform metabolic functions that are essential for the growth and health of the ruminant (Morgavi et al., 2010). Rumen microbes are either anaerobic or facultatively anaerobic, generating end products than can be utilized as an energy source by the host or other microbes (Matthews et al., 2019). Rumen microbial community is affected by several factors such as diet, geographical location, breed, or age (Tapio et al., 2017). The rumen microorganisms can be divided into three main groups: the bacteria, the protozoa and the fungi (Czerkawski, 1986). Methanogens (archaea) are a unique group that has been excluded from the true bacteria and they play an important role in maintaining low hydrogen gas (H_2) in the rumen by using it as a substrate for methane production (Van Soest, 1994b).

2.1 Rumen bacteria

Bacteria are the most abundant prokaryotes found in the rumen, with about 10^9–10^{10} cells per ml of rumen contents accounting for more than 95% of the population in the rumen (McDonald et al., 2011; Cammack et al., 2018; Khalil et al., 2022). The majority are obligate anaerobes, but facultative anaerobes may be present with up to 10^7–10^8 cells/g of rumen contents (Church, 1988). Collectively, rumen bacteria possess a wide

range of enzymatic activities such as amylases, cellulases, proteases, and lipases, which facilitate the digestion of starch, plant cell walls, proteins, and lipids in the rumen (Huws et al., 2018). Classification of rumen bacteria has primarily been based on the type of substrates they can metabolize and on the end products of fermentation they produce. This classification includes groups specializing in cellulose, hemicellulose, starch, sugars, intermediate acids, protein, lipid, or methane production (Church, 1988). An expanded classification may also include ammonia producers and pectin utilizers.

Bacteroidetes and *Firmicutes* are the most common bacterial phyla found in ruminants (Jami et al., 2014; Zeineldin et al., 2018; Clemmons et al., 2019). At the genus level *Prevotella* are the most abundant bacteria in the rumen, accounting for as much as 60% of total cultivable bacteria from the rumen (Bekele et al., 2010; Clemmons et al., 2019), and 90% of *Bacteroidetes* population (Khalil et al., 2022). *Bacteroidetes* are typically in greater abundance in diets consisting primarily of concentrates, while *Firmicutes* are typically in greater abundance in forage-based diets (Clemmons et al., 2019). Jami et al. (2014) reported that the ratio of *Firmicutes* to *Bacteroidetes* was strongly correlated with daily milk fat yield. Despite the vast array of bacterial species estimated to be found in the rumen, only a limited amount has undergone comprehensive investigations.

Among the cellulolytic bacteria in the rumen the most important are *Bacteroides succinogens, Ruminococcus flavefaciens, R. albus,* and *Butyrivibrio fibrisolvens* (Church, 1988). *Ruminobacter amylophilus* and *Prevotella ruminicola* are among the conventional amylolytic bacteria in the rumen. These bacteria produce formate, acetate, and succinate. Other examples include *Selenomonas ruminantium, Succinomonas amylolitica*, and

Streptococcus bovis, which produce acetate, propionate, and lactate as their end products (Russell, 2002). Moreover, major starch degrading (amylolytic) bacteria include *Anaerovibrio lipolytica, Bacteroides amylophilus, Streptococcus bovis, Succinimonas amylolytica,* and *Bacteroides ruminicola* (Church, 1988). Additionally, intermediate VFA-utilizers carry out a secondary fermentation of other rumen bacteria's end products (e.g., lactate, succinate, and formate). For instance, *Megasphaera elsdenii* is an important bacteria used as a probiotic strain to minimize lactic acidosis due to its ability to ferment lactate to acetate, propionate or other larger chain fatty acids (Church, 1988; Weimer & Moen, 2013).

2.2 Rumen protozoa

The protozoa in the rumen are strictly anaerobic and number about 10^5-10^6 cells per ml of rumen contents; they do not often exceed 10^7 cells per ml, but they can account for up to half of the mass (Church, 1988; Russell, 2002; McDonald et al., 2011). Protozoa in the rumen are estimated to account for approximately 2% of the weight of rumen contents, 40% of total microbial nitrogen and 60% of microbial fermentation products in the rumen (Church, 1988). Morphologically the rumen protozoa are divided into ciliates and flagellates (Cammack et al., 2018). Ciliates are being the predominant ruminal protozoal population (Russell & Hespell, 1981). Flagellates are more predominant than ciliates in young calves before developing a ciliate population.

The *Isotrichidae*, and *Ophryoscolecidae* are the two main families of ciliate protozoa that are commonly found in the rumen (McDonald et al., 2011). The *Isotrichidae*, also known as holotrichs, have a shape that is similar to an egg, with various bands of cilia that are distributed evenly on their surface. *Isotrichidae* consists of two

genera, *Isotricha* and *Dasytricha*, which have an important role in breaking down cellulose in the rumen (Russell & Hespell, 1981; McDonald et al., 2011). Conversely, the *Ophryoscolecidae*, or oligotrichs, are a varied group of ciliates with a simple morphology (Russell & Hespell, 1981). This family includes the genera *Entodinium, Diplodinium, Epidinium,* and *Ophryoscolex* (Russell & Hespell, 1981; McDonald et al., 2011). Unlike holotrichs, oligotrichs can consume food particles; but not digest cellulose.

Protozoa, an essential part of the microbial community in the rumen, are known to consume bacteria as a source of protein and compete with them for substrates (Church, 1988). As a result, the number of bacteria in the rumen can be significantly reduced by half or more due to protozoal predation. Although protozoa play a crucial role in the microbial population and have a marked effect on fermentation, the latter has led to controversy in whether they benefit the rumen or not.

2.3 Rumen fungi

Rumen fungi are anaerobic and present in the rumen at concentrations ranging from 10^3 to 10^6 zoospores per ml of rumen fluid (Matthews et al., 2019). They belong to the class *Neocallimastigomycetes* and comprise six previously recognized genera including *Anaeromyces, Caecomyces, Cyllamyces, Neocallimastix, Orpinomyces,* and *Piromyces*. The fungi population is variable, and the role of fungi is defined less accurately. Additionally, *Pichia* is a dominant yeast, the most abundant genus in fungi, and it accounts on average for 64.59 to 91.34 % of fungi (Deng et al., 2020). Despite bacteria being the most prominent microorganisms in the rumen, it is believed that ruminal fungi are more efficient in digesting cellulose and other fibrous materials. This is likely due to their superior enzyme systems as they produce high levels of cellulases and

hemicellulases; and their ability to xylanases to break down xylan (Russell, 2002; Matthews et al., 2019). The production of H_2 by anaerobic fungi during the initial degradation of plant tissue can fuel the degradation mechanisms of other microbial communities, potentially influencing the rest of the rumen microbial community (Matthews et al., 2019).

3. Lactation cycle and transition period in dairy cows

3.1 Lactation cycle

The lactation cycle of dairy cows consists of four main phases: dry period, early lactation, mid-lactation and late lactation (Vijayakumar et al., 2017). During the dry period, cows are not lactating; this is a resting period for the cows to prepare for the upcoming lactation cycle. Early lactation is characterized by a rapid increase in milk production and decreased body conditioning score due to lipid mobilization prompted by a negative energy balance (NEB). The mid and late lactation stages are characterized by steady milk production and a gradual improvement in body condition. Vijayakumar et al. (2017) observed maximum milk yield during the early stage of lactation, indicating that milk production gradually increased up to 90 days and remained high before declining in the later stage of lactation.

3.2 Transition period

The transition period; also called the periparturient period, is typically described as the period starting from 3 weeks prior to calving until 3 weeks after calving (Drackley, 1999). This period consists of complex metabolic adaptations for milk production, and an inadequate metabolic adaptation leads to reduced intake, inflammation, and suppressed

immune functions (Pascottini et al., 2020). Insufficient energy intake; due to reduced intake around parturition, results in the well-known NEB condition (Drackley, 1999; Perez-Baez et al., 2019). Dairy cows commonly experience a degree of NEB during the transition period as they adjust to the heightened energy demands for milk production. However, acute prolonged NEB has detrimental effects on the health and performance of transition cows and predisposes cows to an increased risk of health disorders (e.g. ketosis and displaced abomasum) (Ospina et al., 2010).

Approximately 50-75% of health disorders in dairy cows commonly occur in the first month after parturition, and disease-related culling is a major problem in dairy farms from economic and animal welfare standpoints (LeBlanc et al., 2006; Bradford et al., 2015). Early lactation-related diseases are a significant concern that can potentially comprise the animals' productivity for the entire lactation period. The transition period is a critical stage for high-producing dairy cows. Despite numerous studies on the nutrition and physiology of transition cows, health disorders still occur during this period and have negative economic consequences for the dairy industry. In summary, Trevisi and Minuti (2018) identified five crucial factors linked to the transition period: NEB resulting from increased energy requirements and decreased feed intake, which causes adipose tissue mobilization; decreased immune function; systemic inflammation; oxidative stress; and low blood calcium levels leading to hypocalcemia. Figure 1.2 summarizes the changes in the main physiological aspects of healthy cows during the transition period.

4. Direct fed microbials in ruminants

The terms direct-fed microbials (DFM) and probiotics are often used interchangeably (Quigley, 2011). Fuller (1989) defined the "probiotic" as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Yang et al. (2004) defined DFM as "live, naturally occurring microorganisms that have been used to improve digestive function of livestock". Both terms refer to living microorganisms; these terms are commonly used as synonyms. However, the term probiotic has been used to reference viable microbial cultures, culture extracts, enzyme preparations, and a combination of all three (Yoon & Stern, 1995). Therefore, the US Food and Drug Administration (FDA) redefined the term DFM as "a source of (live) naturally occurring microorganisms" (Yoon & Stern, 1995; Krehbiel et al., 2003).

Direct-fed microbials have three primary ways to impact ruminants, namely as a supplement for silage or haylage, or as a preservative for hay, to replace or reduce the use of antibiotics in stressed cattle, and to improve feed efficiency and increase milk production in dairy cows, as well as body weight gain in beef cattle (Yoon & Stern, 1995). Additionally, several modes of action have been proposed for DFM, including control of rumen pH, enhancement of rumen native microbiota, improved nutrient uptake, and provision of growth factors (Nocek et al., 2003; Nocek & Kautz, 2006; Ban & Guan, 2021). Despite the numerous proposed mechanisms, the specific modes of action of DFM are not fully understood and may vary depending on the type of microorganism used, the dose and duration of administration, and the characteristics of the host animal including diets and ruminal microbiome diversity and abundance (Ban & Guan, 2021). Figure 1.3

summarizes the proposed modes of action of DFM. The DFM can be classified into three categories: bacterial, fungal, and a combination of both (Elghandour et al., 2015).

4.1 Bacterial DFM

The bacterial DFM is the most common and they can be classified as lactic acid producing bacteria (LAB), lactic acid utilizing bacteria (LUB), or other microorganisms (Elghandour et al., 2015). Common microorganisms used as DFM include *Lactobacillus*, *Propionibacterium*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, and *Bacillus*, and rumen derived distinctive bacterial species *Megasphaera elsdenii* and *Prevotella bryantii* (Elghandour et al., 2015; Puniya et al., 2015). Most studies using DFM bacteria have focused on enhancing ruminal lactic acid metabolism through inoculation with LUB bacteria such as *M. elsdenii*, *Selenomonas ruminantium* or *Propionibacterium freudenreichii* (McAllister et al., 2011).

The mode of action of different DFM within the rumen depends mainly on LAB and LUB. LAB, such as *Enterococcus* strains, can have a positive effect on the rumen by preventing ruminal acidosis in dairy cows by facilitating the growth of ruminal microorganisms adapted to the presence of lactic acid in the rumen and by stimulating LUB (Yoon & Stern, 1995; Nocek et al., 2003). LUB, such as M. elsdenii, have been proposed as DFM that can decrease lactate concentrations and maintain ruminal pH (Elghandour et al., 2015). *Propionibacterium* is another lactate-utilizer bacteria naturally found in high numbers in the rumen of animals fed forage and medium concentrate diets (Yang et al., 2004; Elghandour et al., 2015). Certain species of *Propionibacterium* were reported to modify rumen fermentation by fermenting lactate to propionate, the major precursor for gluconeogenesis in the liver of ruminants (Ban & Guan, 2021).

Other bacteria effectively used as DFM are Bifidobacterium sp. and Bacillus sp.(Ullah Khan et al., 2016). Bacillus sp. is known for producing endospores that are resistant to environmental factors and high temperatures, giving them an advantage in surviving during storage or pelleting compared to other bacteria (McAllister et al., 2011). Bacillus sp. are present in the rumen in low numbers and play a minor role in cell wall degradation. Although, strains of Bifidobacterium used as DFM do not come from the rumen, they have a significant effect on digesting starch (Ullah Khan et al., 2016).

4.2 Fungal DFM

Fungal DFM have been widely used in ruminants for improving performance and normalizing rumen fermentation (Elghandour et al., 2015; Puniya et al., 2015). Feeding fungal DFM may improve rumen fermentation by increasing the number and activity of rumen microbes, directly stimulating rumen fungi, and preventing the accumulation of excess lactic acid in the rumen (Puniya et al., 2015). Additionally, (Yoon & Stern, 1996)undertook a review of DFM's in 1995 and concluded that the inclusion of fungal cultures in ruminant diets resulted in various beneficial effects. These included boosting microbial growth, maintaining stable rumen pH, modifying rumen microbial fermentation, enhancing nutrient digestibility, facilitating increased nutrient flow to the small intestine, improving nutrient retention, and alleviating stress.

The yeast, *Saccharomyces cerevisiae,* has gained widespread use as a DFM to enhance the performance and milk production in dairy cattle (Ullah Khan et al., 2016). Moreover, it has been suggested that live yeasts can scavenge oxygen present in the rumen and increase the redox potential of ruminal contents, thereby promoting the growth and activity of anaerobic bacteria (Jiang et al., 2017). Additionally, crude enzyme

extracts of *Aspergillus spp.* fungi are commonly added to ruminant diets to enhance digestion (McAllister et al., 2011). While not technically true DFM, these extracts are intended to enhance fiber or starch digestion in the rumen and can impact feed utilization through various mechanisms. As the extracts are often crude, some likely contain viable fungal cells.

5. Feeding direct-fed microbials to dairy cows

5.1 Effects of direct-fed microbials on lactation performance

Supplementation of DFM to lactating cows has been reported to increase dry matter intake (DMI) (Nocek et al., 2003; Nocek & Kautz, 2006). However, this response is inconsistent as other studies do not report improved DMI in dairy cows (Sun et al., 2013; Goldsmith et al., 2023); but reported improvements in feed efficiency. An increase in output without a corresponding increase in dry matter intake (DMI) suggests that animals utilize more nutrients from the same amount of consumed DM, improving feed efficiency. Nocek et al. (2003); AlZahal et al. (2014) support this idea by reporting that feeding DFM enhances nutrient digestibility.

Direct-fed microbials have been reported to increase milk yield in dairy cows without affecting milk composition, although responses may vary over time. Kumprechtová et al. (2019) observed an increase of 1.6 kg/d in milk yield for early lactation cows supplemented with live yeast *S. cerevisiae.* Similarly, Nocek and Kautz (2006) reported an even greater increase in milk yield of 2.3 kg/d with a combination consisting of 2 strains of *Enterococcus faecium* and *S. cerevisiae.* Additionally, Boyd et al. (2011) supplemented a combination of *Lactobacillus acidophilus* and

Propionibacterium freudenreichii to mid-lactation cows and observed an increase in milk yield and energy-corrected milk; but no effect on milk composition.

Conversely, several studies have reported no effect of DFM supplementation on milk production and composition. For instance, Bayat et al. (2015) supplemented during early lactation 2 strains of *S. cerevisiae* and reported no treatment effect on milk, fat and protein yield. Similarly, a study that fed a combination of *Propionibacterium sp., Lactobacillus plantarum,* and *Lactobacillus rhamnosus* to early lactating cows observed no milk yield improvement and no effect on milk fat and protein. Conversely, (Oetzel et al., 2007) observed increased milk fat concentration for primiparous cows and an increase in milk protein percentage for the second lactation cows using DFM of 2 strains of lactic producer *Enterococcus faecium* plus *Saccharomyces cerevisiae* yeast.

5.2 Effects of direct-fed microbials in the transition period

As stated before, lactating dairy cows go through NEB during transition period characterized by fat mobilization from adipose tissue to meet the energy requirements to sustain milk production. Nocek et al. (2003) reported that cows supplemented with *Enterococcus faecium* had higher blood glucose and insulin levels postpartum, indicating the availability of more energy. Additionally, these cows had lower levels of non-esterified fatty acids (NEFA), suggesting better metabolic health. This aligns with Kumprechtová et al. (2019), who reported lower blood NEFA levels after calving in cows supplemented with live yeast *s. cerevisiae*.

A lower concentration of NEFA indicates that cows are mobilizing less fat. According to Nocek and Kautz (2006), cows consuming a combination of *S.cerevisiae*

and 2 strains of *Enterococcus faecium* had a lower concentration of β-hydroxybutyrate after calving, which suggests that supplementing DFM can reduce the amount of energy cows take from adipose tissue. Additionally, Luan et al. (2015) found that cows consuming Bacillus pumilus as a DFM had less subclinical ketosis after calving. This implies that supplementing DFM may help decrease the energy cows need to mobilize from adipose tissue. When blood glucose is made available and cows are mobilizing less fatty acids from adipose tissues, glucose can go to the mammary gland to produce more milk. Overall, these findings suggest that DFM have the potential to make the diet more energetically favorable for cows during the transition period.

6. Use of rumen-derived *Clostridium beijerinckii, Pichia kudriavzevii, Ruminococcus bovis,* and *Butyrivibrio fibrisolvens* as Direct-fed microbial in dairy cows.

In simulated rumen conditions, *Pichia kudriavzevii*, a budding fungus, has demonstrated the ability to break down complex polysaccharides and cellulose, as reported by Suntara et al. (2021) and Valldecabres et al. (2022). On the other hand, *C. beijerinckii* is a bacteria that cannot degrade cellulose (Gomez-Flores et al., 2017). However, according to Gomez-Flores et al. (2017), *C. beijerinckii* can produce acetate and butyrate when co-cultured with other microorganisms that can break down complex carbohydrates. *B. fibrisolvens* is a well-known bacterium that plays a crucial role in the biohydrogenation process in the rumen (Amin & Mao, 2021). Gaffney et al. (2021) reported recently that *R. bovis* is capable of degrading rumen undegradable starch with acetate as the main fermentation end-product. All four microorganisms mentioned above were originally isolated, cultured and converted into a dried product DFM from the rumen of high-performing Holstein dairy cows.

There is currently a lack of research investigating the supplementation of rumen-derived DFM containing *C. beijerinckii, P. kudriavzevii, R. bovis,* and *B. fibrisolvens* in transition cows. Previous studies have only examined their effects on performance in early and mid-lactation cows and did not report data on blood biomarkers and rumen fermentation profile, and microbial abundance (Goetz et al., 2021; Dickerson et al., 2022; Valldecabres et al., 2022; Goldsmith et al., 2023). Dickerson et al. (2022) and Goldsmith et al. (2023) observed no treatment effect on milk yield or milk components. However Dickerson et al. (2022) observed a treatment over time interaction for greater energy corrected milk (ECM) in DFM supplemented cows. Similarly, Valldecabres et al. (2022) reported increased milk yield (+2.9 kg/d) and ECM (+3.1kg/d) in DFM supplemented cows, as well as greater fat and protein yield.

RATIONALE AND OBJECTIVES

The overall goal of this study was to understand the physiological responses to feeding a rumen derived DFM supplement to transition dairy cows until 100 days in milk. Therefore, the objectives of this study were to evaluate the effects of a commercial rumen derived direct-fed microbial product on performance, blood biomarkers, rumen fermentation, and bacterial abundance.

CONCLUSION

The success of a lactation cycle in dairy cows depends on the success of the transition period, as stated by Drackley (1999). To ensure a successful transition, appropriate nutrition and management practices are crucial (Drackley et al., 2005). The transition period is known to pose metabolic and immunological challenges, such as NEB

resulting from decreased DMI around parturition and mobilization of adipose tissue. These factors should be considered when designing dry cow management. Additionally, during early lactation, dairy cows are at a higher risk of developing metabolic disorders, and management approaches should prioritize preventing such disorders.

Research has led to the development of nutritional strategies to aid cows in transitioning smoothly from late pregnancy to lactation. One such strategy is the use of direct-fed microbials in the cows feed, which have gained popularity in recent years. The use of these products has been found to be beneficial in terms of improving both performance and health. Direct-fed microbials can be comprised of bacteria, fungi or a combination of both. Despite the numerous proposed mechanisms, the specific modes of action of DFM are not fully understood and may vary depending on the type of microorganism used (e.g., bacterial or fungal) in DFM, the dose and duration of administration, and the characteristics of the host animal including host-microbiome composition (Ban & Guan, 2021).

Although there is growing interest in using DFMs as a nutritional strategy to promote the health and performance of dairy cows during the transition period, there is still limited research on the effects of these products. Specifically, more studies are needed to understand the mechanisms by which DFMs influence the health of transition dairy cows and to optimize their use for the best outcomes. While there is a vast range of commercial DFM products available, their specific impacts on the metabolism and immune system of dairy cows during the transition period are not yet fully elucidated. Therefore, further research is needed to fully comprehend the potential benefits of DFMs

in transition dairy cows and to maximize their use for improved productivity and profitability in commercial dairy farms.

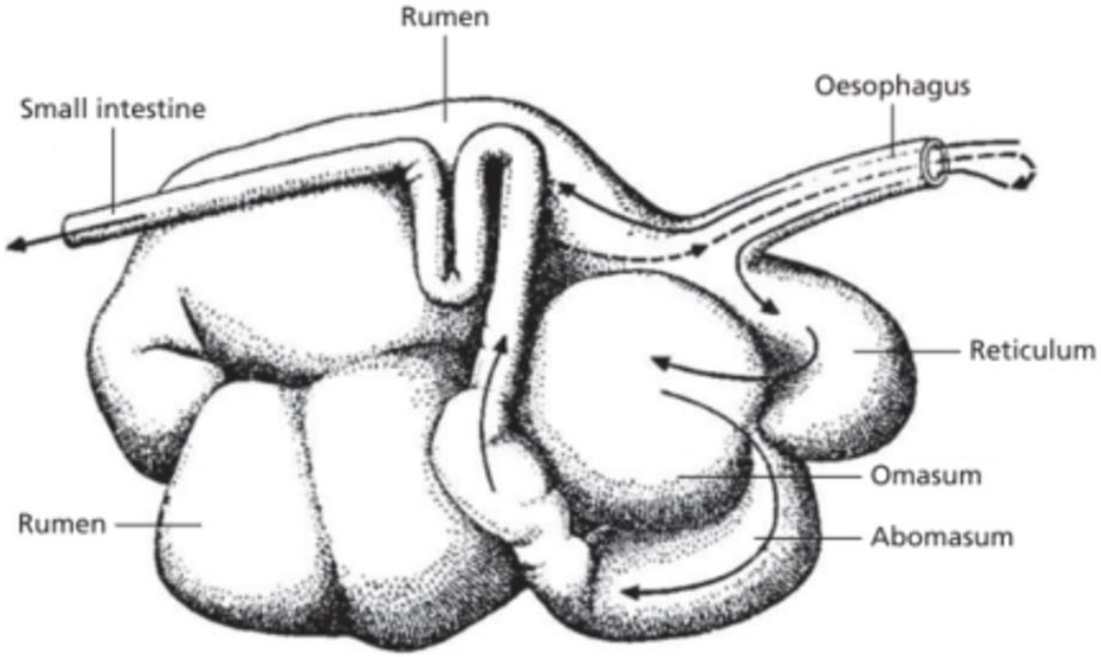

Figure 1.1 - Diagrammatic representation of the rumen, reticulum, omasum and abomasum of the ruminant, indicating the flow of digesta. Source: (McDonald et al., 2011).

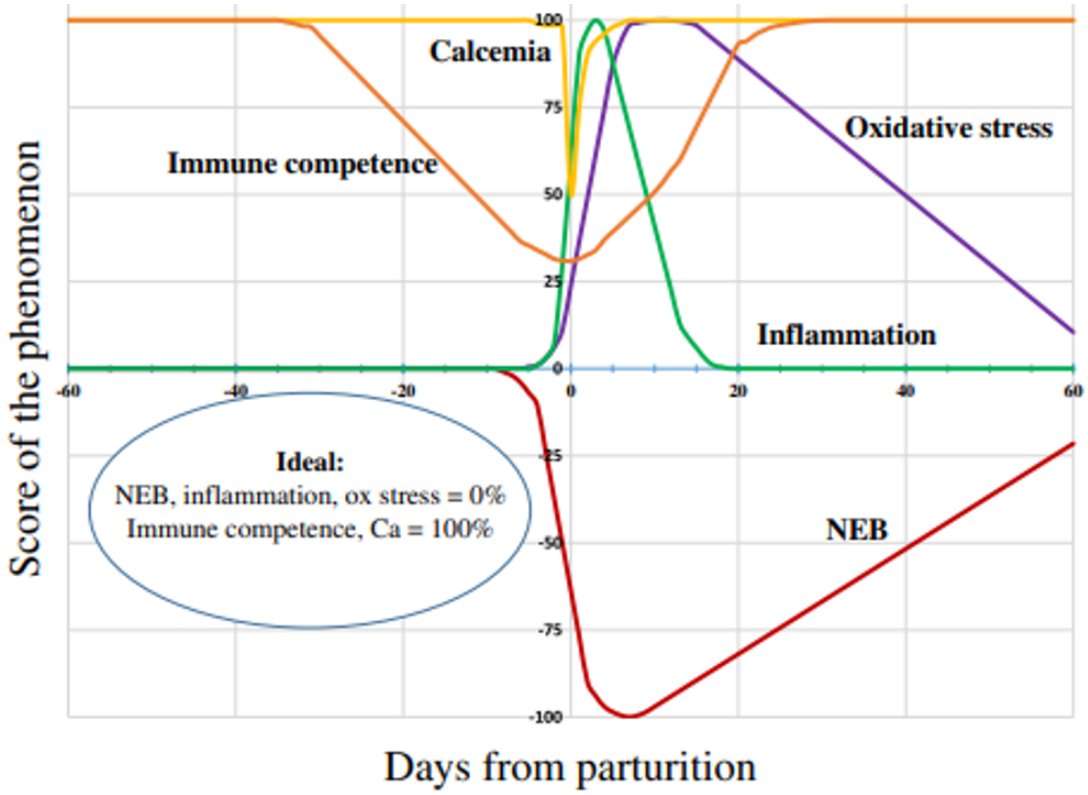

Figure 1.2- Theoretical pattern of changes in the main physiological aspects of healthy subjects during the transition period. Ideally, the Negative energy balance (NEB), inflammation, and oxidative stress would be close to zero (i.e. absence of the phenomena), whereas the immunocompetence and the calcemia would be close to 100% of their optimal level. Source:(Trevisi & Minuti, 2018)

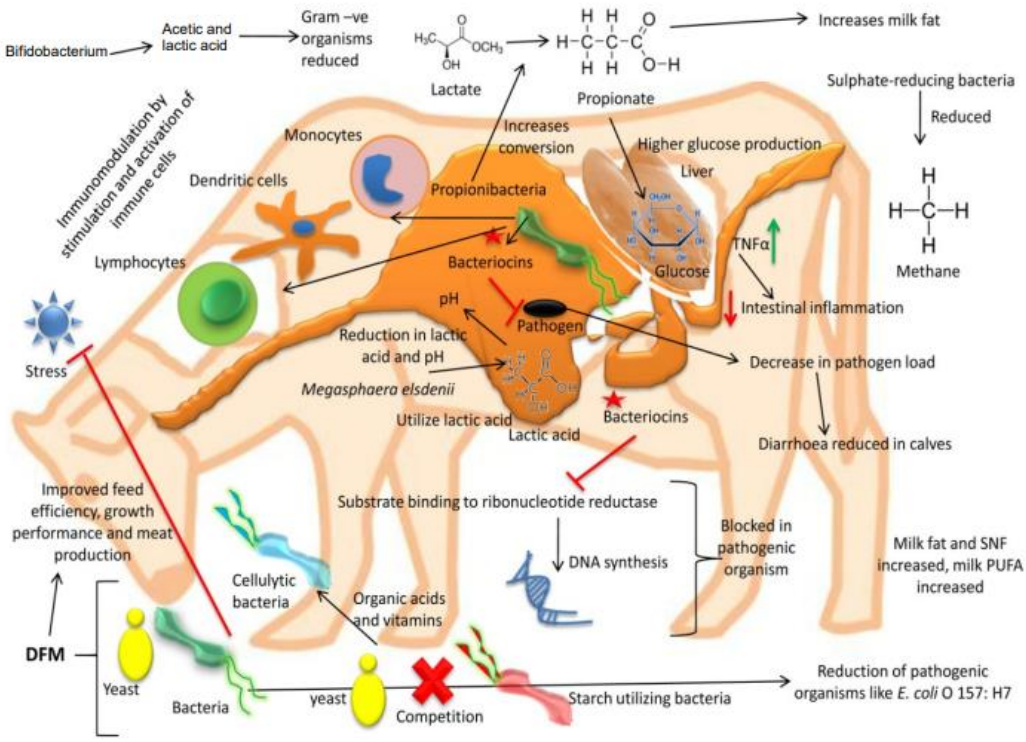

Figure 3.3 - An overview of modes of actions and beneficial applications of DFM for enhancing ruminant production and protecting health. Source: (Ullah Khan et al., 2016)

CHAPTER 2. *IN-VIVO* ASSESSMENT OF A DIRECT-FED MICROBIAL ON LACTATION PERFORMANCES, BLOOD BIOMARKERS, RUMINAL FERMENTATION AND MICROBIAL ABUNDANCE IN HOLSTEIN COWS.

ABSTRACT

The objective of this study was to evaluate the effects of a rumen-derived direct-fed microbial (DFM) on performances, blood biomarkers, rumen fermentation profile, and bacterial abundance in dairy cows during the transition period to mid-lactation, until 100 d in milk (DIM). Fifty-six Holstein cows were enrolled in a randomized complete block design from -21 to 100 DIM and blocked based on expected calving date, parity, and previous lactation milk yield or genetic merit. At -21 DIM, cows were randomly assigned to a control (CON, basal diet+150 g/d of ground corn; n=29) or treatment group (GF, basal diet + 150 g/d of ground corn + 5g/d of DFM; n=27), top-dressed once a day. All cows received the same basal close-up diet from -21 DIM until calving (0.71 Mcal/lb and 14.46% crude protein, CP) and lactation diet from calving to 100 DIM (0.80 Mcal/lb and 15.69% CP). Blood samples were collected for biomarkers of metabolism, inflammation, and oxidative stress whereas rumen fluid for ammonia, volatile fatty acids (VFA), and microbial abundance from a subset of multiparous cows (n=12/Treatment) at various time points from -22 to 100 DIM. Statistical significance and tendency were declared as $P < 0.05$, and $P \geq 0.05$ and $P < 0.10$, respectively. Compared to CON, GF cows tended to produce greater milk (+2.64 kg/d) during mid-lactation (31 to 100 DIM). Although DM intake (DMI) was not affected by the treatment, GF cows tended to have greater feed efficiency (+0.11, milk/DMI) in early lactation (0 to 30 DIM). Compared to CON, GF cows had lower blood plasma glucose and higher BHB (P=0.03). Inflammation biomarkers

showed greater concentrations of ceruloplasmin and haptoglobin in GF cows than CON. Compared to CON, GF cows had greater ruminal butyrate and tended to have greater valerate and lower acetate, which was coupled with alterations in rumen microbial abundance, showing a greater abundance of lactate-utilizing (*Megasphaera elsdenii*) but lower abundance of cellulose-utilizing species (*Fibrobacter succinogens*) compared to CON. Overall, GF supplementation during the transition period may have potential benefits for rumen environment and performance, but the increased inflammation after calving requires further investigation.

Key words: probiotics, transition cow, rumen microbiome

INTRODUCTION

During the transition period, defined as the last 3 wk before parturition to 3 wk after parturition (Drackley, 1999), cows undergo drastic adaptive physiological, metabolic and immunological changes (Bernabucci et al., 2005; Drackley et al., 2005). A negative energy balance, resulting from an inadequate feed or energy intake in response to meeting the high energy demand for milk synthesis, usually leads to body condition score loss, higher risk of metabolic disorders, and alterations in immune function (Halfen et al., 2021; Hiltz et al., 2023). Immune dysfunction and metabolic stress increase the likelihood of developing health disorders during early lactation (Carpinelli et al., 2023; Mezzetti et al., 2020). Overall, the parturition transition imposes a significant metabolic and immunologic stress which needs to be managed to optimize cattle health and productivity (Hiltz et al., 2023).

Direct-fed microbials (DFM) are feed additives commonly supplemented via diet to improve production, efficiency and health in dairy cows (Goldsmith et al., 2023). The term direct-fed microbial has been previously defined as “live, naturally occurring microorganisms that have been used to improve digestive function of livestock” (Yang et al., 2004). The DFM can be classified into three categories: bacterial, fungal, and combination of both (Elghandour et al., 2015). Several modes of action have been proposed for DFM, including control of rumen pH by stimulation of lactic acid utilizing bacteria, enhancement of rumen native microbiota, improved nutrient uptake, and provision of growth factors (Ban & Guan, 2021; Nocek & Kautz, 2006; Nocek et al., 2003). Despite the numerous proposed mechanisms, the specific modes of action of DFM

are not fully understood and may vary depending on the type of microorganism used (e.g. bacterial or fungal), the dose and duration of administration, and the characteristics of the host animal including diets and ruminal microbiome diversity and abundance (Ban & Guan, 2021).

Several studies have reported the positive effects of supplementing DFM on the performance of dairy cows during transition period, as well as in early-and mid-lactation (Hiltz et al., 2023; Kumprechtová et al., 2019; Nocek & Kautz, 2006; Oh et al., 2019). Moreover, some studies have investigated the influence of these feed additives on rumen fermentation (Chiquette et al., 2015; Mamuad et al., 2019; Philippeau et al., 2017; Weiss et al., 2008), energy metabolism (AlZahal, McGill, et al., 2014; Luan et al., 2015; Oetzel et al., 2007), immune status, and inflammation responses (Hiltz et al., 2023). However, these conventional DFM are not native to the rumen and have a limited ability to manipulate and interact with the rumen and its native microbial community (Goetz et al., 2021). Current literature on supplementation of rumen-derived DFM is scarce and has been evaluated in mid-lactating cows solely (Dickerson et al., 2022; Goetz et al., 2021; Goldsmith et al., 2023; Valldecabres et al., 2022).

In this study, we evaluated a DFM comprised of 3 bacterial species (Clostridium beijerinckii, Ruminococcus bovis, and Butyrivibrio fibrisolvens) and 1 fungal species (Pichia kudriavzevii), that were originally isolated, cultured and converted into a dried product from the rumen of high-performing Holstein dairy cows. Pichia kudriavzevii is a budding fungus that produces cellulase and has been reported to degrade cellulose and other complex polysaccharides in in-vitro studies simulating rumen conditions (Suntara et al., 2021; Valldecabres et al., 2022). Conversely, C. beijerinckii is not able to degrade

cellulose, however, it has been reported to produce butyrate and acetate when co-cultured with other complex carbohydrate degraders (Gomez-Flores et al., 2017). Additionally, B. fibrisolvens is a well-known bacteria considered as the main one responsible for the biohydrogenation process in the rumen (Amin & Mao, 2021) while R. bovis is a novel species able to degrade rumen undegradable starch with acetate as major fermentation end-product (Gaffney et al., 2021). Therefore, we hypothesized that supplementing this rumen-derived DFM would influence lactation performances and rumen environment. The objective of this study was to evaluate the effects of a rumen-derived DFM [Galaxis™ Frontier (GF); Native Microbials, Inc., CA, USA] on performances, blood biomarkers, rumen fermentation profile, and bacterial abundance in dairy cows from transition period until 100 DIM.

MATERIAL AND METHODS

Experimental Design and Dietary Treatments

All the procedures for this study were approved by The Institutional Animal Care and Use Committee (IACUC) of South Dakota State University (Protocol # 2011-053A). Fifty-six Holstein cows (43 multiparous and 13 primiparous) were enrolled in a randomized complete block design study at -21 d relative to calving and remained on the study until 100 DIM. Cows were blocked as per expected calving date, parity, and previous lactation milk yield for multiparous or genetic merit for primiparous cows. All cows received the same close-up diet during the dry period (0.71 Mcal/lb and 14.46% CP) and the same lactation basal diet after parturition from calving to 100 DIM (0.80 Mcal/lb and 15.69% CP). Diets were fed as total mixed rations (TMR) and cows had ad

libitum access to drinking water. At -21 days prior to expected calving date, cows were assigned either to control (CON; basal close-up diet + 150 g/d ground corn, n= 29) or treatment group (GF; basal close-up diet + 150 g/d ground corn + 5 g/d GF; n=27). Ground corn (Placebo) and ground corn containing GF were fed to cows as top-dressed in CON and treatment group, respectively. Upon calving, all cows regardless of control or treatment group were transitioned to same lactation diet as basal diet.

Animal Management and Body weight

Cows were enrolled in the study from mid-April to mid-September 2021. Cows were individually fed using Calan gate system (American Calan, Inc.; Northwood, NH), and individual feed intake was recorded daily. During the dry period, cows were housed in a bedded-pack pen until parturition, whereas they were relocated into individual pen upon parturition and stayed there until 3 DIM. Three days after calving, cows were moved to a free-stall lactating barn, where they received the same lactation diet as basal until 100 DIM postpartum.

Body weight was measured weekly for each cow at 1200 h. Body condition using 1 to 5 point scoring system (1 = emaciated, 5 = obese) was recorded weekly by two individuals, and the average score was used for statistical analysis.

Feed and Milk Samples

DM content of individual ingredients was determined weekly (100˚C for 24 h), and diets were adjusted to maintain DM ratio of ingredients in TMR. Individual sample of each ingredient and TMR were collected weekly and stored at -20˚C until further analysis. Each ingredient was then composited into monthly sample and analyzed for DM (100˚C for 24h), CP (AOAC Official Method 990.03 for forages and AOAC Official

Method 992.23 for grain and cottonseed), NDF (ANKOM Technology Method 15), and ADF (ANKOM Technology Method 14) and NEL was calculated (Dairy One, Ithaca, NY; https://dairyone.com/download/forage-forage-lab-analytical-procedures/).

Cows were milked twice daily at 0530 h and 1630 h, and AM & PM milk yield were recorded daily until 100 DIM. Consecutive morning (AM) and evening (PM) milk samples were collected weekly during the experimental period. Milk samples were preserved with (Broad Spectrum Microtabs II, Advanced Instruments) and analyzed separately as AM and PM sample for milk fat, protein, somatic cell count (SCC), milk urea nitrogen (MUN), lactose and solids. The ECM was calculated based on milk yield and milk sample analysis as follows: ECM = [12.82 × fat yield (kg)] + [7.13 × protein yield (kg)] + [0.323 × milk yield (kg)] (Hutjens, 2010). Equations from NRC (2001) were used to calculate energy balance (EB) for each cow. The energy intake was determined using daily DMI multiplied by NEL density of the diet. Net energy of maintenance was calculated as $BW^{0.75} \times 0.080$. Requirements of NEL were calculated as NEL = (0.0929 × fat % + 0.0547 × protein % + 0.0395 × lactose %) × milk yield. The net energy requirement for pregnancy (NEP; Mcal/d) was calculated as NEP = [(0.00318 × day of gestation − 0.0352) × (calf birth weight/45)]/0.218. The equation used to calculate prepartal EB (EBPRE; Mcal/d) was EBPRE = NEI − (NEM + NEP) and EBPRE (as % of requirements) = [NEI/ (NEM + NEP)] × 100. The equation used to calculate postpartum EB (EBPOST) was EBPOST (Mcal/d) = NEI − (NEM + NEL) and EBPOST (as % of requirements) = [NEI/ (NEM + NEL)] × 100.

Blood Collection and Analyses

Blood was sampled from the coccygeal vein before morning feeding using a 20-gauge needle (Beckton Dickinson, Franklin Lakes, NJ) twice a week from -22 days until calving and three times a week after calving until 30 DIM. Blood was collected into evacuated tubes (5 mL, BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing either clot activator for serum samples or heparin for plasma samples. After blood collection, tubes for plasma were placed on ice and tubes for serum were kept at 21˚C until centrifugation (~30 min). Serum and plasma were obtained by centrifugation at 1300 × g for 15 min at 21˚C and 4˚C, respectively. Aliquots of serum and plasma were stored at -80˚C until further analysis.

Plasma samples of -22, -14, -7, 7, 14 and 30 days relative to calving were analyzed for biomarkers of energy metabolism [i.e., glucose, non-esterified fatty acids (NEFA), and BHB)], protein and/or N metabolism (i.e., urea, protein, and creatine), liver function [i.e., total bilirubin, glutamic oxaloacetic transaminase (GOT), gamma-glutamyl transferase (GGT), cholesterol, paraoxonase, and albumin], inflammation (i.e., ceruloplasmin, haptoglobin, and globulin), and oxidative stress (i.e., reactive oxygen metabolites and ferric reducing antioxidant power) using Instrumentation Laboratory Kits following the procedures described by (Trevisi et al., 2012; Batistel et al., 2016; Jacometo et al., 2016).

Collection and Analyses of Rumen Fluid

Rumen fluid was collected 3-4 hr after feeding on day -22, -14, -7, 1, 7,14, 21, 70 and 90 relative to parturition from a subset of multiparous cows (n = 12/ treatment) via esophageal tubing. The first 20 mL of fluid was discarded to minimize saliva

contamination and approximately 50 mL sample was collected from each cow. Rumen pH was measured immediately after collection using a pH meter (Oakton Instruments, Vernon Hills, IL) to verify the quality of the samples. Two aliquots of 10 mL were saved containing either 200 µL of 50% sulfuric acid for ammonia (NH3) or 2 mL of 25% metaphosphoric acid for VFA and stored at -20˚C until further analysis. Additionally, 2 mL of rumen fluid samples that were immediately placed on ice after sampling and stored in liquid nitrogen until DNA isolation, were analyzed for relative abundance of bacteria species quantification using qPCR technique.

Rumen fluid samples for NH3 were thawed and transferred into a 2mL microcentrifuge tube and centrifuged at 30,000 × g for 20 min at 4°C (Model 5403, Eppendorf, Hamburg, Germany). The supernatant of rumen fluid sample was used to analyze NH3-N concentration using the assay described by Chaney and Marbach (1962). For the analysis of VFA concentrations thawed rumen fluid samples (1 ml) were acidified with 0.17 mL of metaphosphoric acid (25%, w/v), and 0.13 mL of internal standard (5 mmol, 4-methyl-valeric acid, Sigma, St. Louis, MO), vortexed, and let it rest for 30 min (4 °C). Then samples were centrifuged at 3000 × g for 15 min. The supernatant was collected and used for VFA determination using a 6890 N Network GC System gas chromatograph (Agilent Technologies) equipped with a flame ionization detector, according to (Izuddin et al., 2019). One microliter of the sample was injected at split 1:30, at a temperature of 230 °C. Separation of VFA profile was determined using Quadrex 007-10 Series (Quadrex Corp., New Haven, CT 06525, USA) bonded phase fused silica capillary column (15 m, 0.250 mm internal diameter, 0.25 µm film thickness). The temperature of the column was set at 60 °C and held for 2 min; increased

to 100 °C (10 °C/min), increased to 200 °C (20 °C/min), and held for 5 min. Nitrogen gas was supplied as carrier gas at the rate of 1 mL/min. The temperature of the detector was set at 230 °C. Commercial standards (Sigma-Aldrich, St. Louis, MO) of acetic (45997), propionic (94425), iso-butyric (46935), butyric (19215), iso-valeric (78651), valeric (75054), and caproic (21529) acids were used as external standards for peak identification. The molar concentration of VFA was identified based on a single point of internal standard and calibration curve with external standards.

Isolation and Amplification of Ruminal Fluid Bacterial DNA using qPCR

The DNA of ruminal bacteria was obtained using the QIAamp Fast DNA Stool mini kit from (Qiagen, Hilden, Germany) with some modifications to the protocol outlined by (Tapio et al., 2017). Initially, 1 mL of rumen fluid was centrifuged at 12,000 × g for 5 min at 20-25°C. After discarding supernatant, the pellet was suspended in 1 mL of buffer EX, vortexed, and incubated at 95°C for 5 min, and then centrifuged at 20,000 × g for 1 min at 20-25°C. Afterward, 600 µL of the supernatant was transferred to a new microcentrifuge tube containing 25 µL of Qiagen proteinase K, followed by the addition of 600 µL of buffer AL. The mixture was vortexed for 15s and incubated at 70°C for 10 min. Subsequently, 600 µL of 96% molecular ethanol was added and vortexed. The mixture was transferred into a QIAamp mini spin column, and the manufacturer's procedures were followed. A NanoDrop spectrophotometer (ND 1000, NanoDrop Technologies Inc., Wilmington, DE) was used to determine the quantity and purity of the extracted DNA, which was standardized to 8 ng/ µL for qPCR.

The primer sets used in the study were previously validated and reported (Table 2.2) The relative abundance of 18 bacterial species was determined using qPCR analysis. The

qPCR analysis was performed using 10 µL of qPCR mixture containing 4 µL of sample DNA, 5 µL of 1 x SYBR Green master mix (Applied Biosystems, Waltham, MA), 0.4 µL of 10 µM each for forward and reverse primers, and 0.2 µL of DNase-RNase-free water in a MicroAmp Optical 384-well reaction plate (Applied Biosystems). Each sample was run in triplicate, and the relative abundance was determined based on a 6-point standard curve plus a no-template control. The 4-fold-dilution standard curve was created using standardized DNA from all samples. The qPCR reactions were performed with the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). To determine the relative abundance of bacterial species, the efficiency-corrected Δ-CT method (Abdelmegeid et al., 2018) was employed, using the geometric mean of 2 universal primers. The relative abundance of each bacterial species is relative to the total bacteria abundance measured with universal primers.

Statistical Analysis

Production data were analyzed separately for early lactation (0-4 weeks postpartum) and mid lactation (5-14 weeks postpartum) periods. Data were analyzed as repeated measures with MIXED procedure of SAS (SAS Institute Inc.) with the following model:

$$Y_{ijklm} = \mu + D_i + P_j + DP_{ij} + B_k + C_{ijkl} + T_m + DT_{im} + DPT_{ijm} + e_{ijklm}$$

Where Y_{ijklm} the dependent, continuous variable; µ is the overall mean; D_i is the fixed effect of the ith diet (i = 1 and 2); $P_j$is the fixed effect of the jth parity (j = 1, 2, 3); $B_k$is the random effect of the kth block (k = 1, …20); $C_{ijkl}$is the random effect of lth cow nested within the ith treatment, the jth parity, and the kth block (l = 1,…, n_ijk); T_m is the fixed effect of the mth time (daily for rumen fluid and biomarker parameters or weekly

for DMI, BCS, BW, milk yield and milk composition parameters) of the experiment (m = 1,… n); $DT_{im}$is the fixed effect of the ith treatment by the mth time of the experiment interaction; $DPT_{ijm}$is the fixed effect of the ith treatment by the jth parity by the mth time of the experiment interaction; and $e_{ijklm}$is the residual error. Parity and block effects were removed from the model any time it was nonsignificant (P > 0.05). Blood biomarkers and rumen fluid data, including pH, VFA, NH3, and relative abundance of microbial species were analyzed at various time points that were not equally spaced; therefore, an exponential correlation covariance structure SP (POW) was used for repeated measures. Blood biomarkers and relative abundance of microbial species were log-scale transformed if needed to comply with normal distribution of residuals assumption. Blood biomarkers and rumen fluid data on -22 DIM were used as a covariate. The covariate of previous 305-d milk yield was maintained in the model for all variables for which it was significant (P < 0.05). Statistical significance was declared at P <≤ 0.05 and tendencies at P ≥ 0.05 and < 0.10.

RESULTS

DMI, BW, BCS, and EB

When p-values presented only correspond to treatment, time and treatment × time interaction is because all other interactions were not significant or did not show tendency. Main effects and interactions for prepartum and postpartum BW, BCS, DMI, and EB are presented in Table 2.3. Neither prepartal nor postpartal BW, BCS, DMI, and EB were affected (P ≥ 0.17) by dietary treatments. Explain treatment by time interaction tendency for DMI.

Production Variables and Feed Efficiency

Main effects and interactions for postpartal production variables and feed efficiency (milk production/DMI) are presented in Table 2.4 Milk production did not vary between treatment (GF) and CON group when analyzed milk data collected from whole study period (P = 0.13), however, milk production tended to be greater in GF than CON during mid-lactation period (5-14 wk postpartum; P = 0.08; Table 2.4) where GF produced 2.64 kg/d more than control cows. Similarly, feed efficiency (milk/DMI) did not differ between groups (P = 0.16) when analyzed whole study period however feed efficiency during early lactation (0-4 wk postpartum) tended (P = 0.10) to be greater in GF than CON cows (Figure 2.1 D). There was a treatment × time interaction (P < 0.01) for milk fat and protein percentage (Figure 2.2), where milk fat (%) was lower (P<0.01) in GF cows in comparison to control at wk 11; Figure 2.2A. Milk protein (%) was greater (P = 0.04) at wk 1, but lower (P = 0.04) at wk 9 and 13 in GF than CON cows Figure 2.2B. Treatment did not affect MUN, SCC, and ECM.

Rumen Fermentation

Main effects and interactions for rumen fermentation characteristics are presented in Table 2.5. There was a Treatment x time interaction observed for butyrate, which was attributed to greater (P = 0.02) butyrate proportion in GF than CON cows at 1 and 70 DIM (Figure 2.3). Likewise, there was a treatment effect for greater (P = 0.04) butyrate proportion in GF than CON cows. Additionally, we observed a trend for lower (P = 0.10) acetate proportions and greater (P = 0.06) valerate proportions in GF than CON cows. Treatment did not affect ruminal pH, NH3, and total VFA production.

Abundance of Abundance of Ruminal Bacteria

Main effects and interactions for relative abundance of selected bacterial species are presented in Table 2.6. We found a tendency of treatment x time interaction for (P = 0.08). *Anaerovibrio lipolytica* (Table 2.6). *Anaerovibrio lipolytica* tended to show greater abundance in CON than GF cows at 7 and 100 DIM (Figure 2.4A). Additionally, a tendency for greater abundance of *Megasphaera elsdenii* (P = 0.07) and *Prevotella albensis* (P = 0.09) was observed in GF than CON cows. In contrast, *Fibrobacter succinogens* tended to be in lower abundance in GF than CON cows (P = 0.07).

Blood Biomarkers of Energy and Nitrogen Metabolism

Main effects and interactions for blood biomarkers related with energy metabolism and nitrogen metabolism are presented in Table 2.7. Among biomarkers related to energy metabolism, glucose was lower (P = 0.03) in GF than CON cows throughout the experiment and reached nadir levels at 7 d postpartum (Figure 2.5 A). Blood plasma BHB was greater (P = 0.03) in GF than CON cows. Additionally, a parity x treatment interaction (P <0.01) observed for glucose was reflected as lower (P = 0.01) glucose levels in GF than CON cows during second lactation. Similarly, a parity x treatment interaction (P = 0.01) was observed for BHB, in which GF cows had greater (P = 0.05) BHB levels than CON cows in second lactation. Blood NEFA was mainly affected by time (P <0.01) and reached peak l at 7 d postpartum (Figure 2.5). Treatment did not affect nitrogen metabolism related biomarkers such as blood concentrations of urea, protein, and creatinine.

Blood Biomarkers of Liver Function

Main effects and interactions for blood biomarkers related to liver function are presented in Table 2.7. We did not find any significant treatment × time interaction. Among the biomarkers related to liver function, only paraoxonase were affected by the treatment (P =0.03) being for lower in GF than CON cows and reached nadir level at 7 d postpartum (Figure 2.6A). The remaining blood biomarkers related to liver function exhibited statistically significant temporal effects (P < 0.01).

Blood Biomarkers of Inflammation and Acute-Phase Proteins

Main effects and interactions for inflammation and acute-phase proteins (APP) biomarkers are presented in Table 2.7. None of the treatment × time were significant (P = 0.05) for inflammation or APP biomarkers variables measured. Unlike globulin, GF cow had a greater concentration of ceruloplasmin (Figure 2.6 B; P = 0.01) and haptoglobin (Figure 2.6 C; P = 0.02) than CON cows.

Blood Biomarkers of Oxidative Stress

Main effects and interactions for biomarkers related with oxidative stress are presented in Table 2.7. None of the treatment × time were significant (P = 0.05) for oxidative stress biomarkers. In contrast to FRAP, which was only impacted by the time (P<0.01), ROM exhibited a significantly greater concentration in GF than CON cows (Figure 2.6 D; P = 0.01).

DISCUSSION

Effects on DMI, BW, BCS, and EB

In the current study, GF supplementation did not affect prepartal and postpartal DMI, BW, BCS and EB. Our results agree with those of Goetz et al. (2021), who also

reported a greater DMI in cows fed DFM, but with no statistical difference compared to the control group. It should be noted, however, that Goetz et al. (2021) study used a DFM comprised solely of *C. beijerinckii* and *P. kudriavzevii*. It is widely recognized that peripartal dairy cows commonly experience a reduction in DMI around parturition (Drackley, 1999). This decline in DMI is related to physical, behavioral, metabolic and hormonal changes around parturition (Contreras & Sordillo, 2011). Insufficient energy intake both before and after parturition results in a condition known as negative energy balance (NEB), wherein lipids from adipose tissue are released in the form of non-esterified fatty acids (NEFA) (Perez-Baez et al., 2019). When energy is inadequate to support the maintenance, gestation, or lactation requirements, insufficient energy intake from feed can lead to a decline in BW, a decrease in BCS, or both (Gross et al., 2011).

Conventional direct-fed microbials (DFM) have been utilized in the supplementation of transition dairy cows, typically containing *Saccharomyces cerevisiae*, with the aim to enhance their cows' health and performances. (Oetzel et al., 2007). For instance, Nocek et al. (2003) reported increased DMI throughout the first 21 days of lactation when feeding a DFM product containing two specific strains of *Enterococcus faecium* and yeast to transition Holstein dairy cows. However, these conventional DFM consist of microbial strains that are not native to the rumen. There is currently a lack of research investigating the supplementation of rumen-derived DFM containing *Clostridium beijerinckii, Pichia kudriavzevii, Ruminococcus bovis,* and *Butyrivibrio fibrisolvens* in transition cows, as previous studies have only examined their effects in early and mid-lactation cows (Dickerson et al., 2022; Goetz et al., 2021; Goldsmith et al., 2023; Valldecabres et al., 2022).

observed that in early lactating cows' diet supplemented with DFM at a rate of 0.33g/kg of TMR of the same DFM forementioned had a greater DMI. Conversely, a study conducted at Michigan State University indicated a trend toward decreased DMI in mid-lactation cows supplemented with who were provided the same DFM (Goldsmith et al., 2023). Similarly, Dickerson et al. (2022) noted a numerical decrease in DMI among the DFM groups, although not statistically different from control.

Milk production and milk composition

Recent studies have investigated the effects of supplementing cows with rumen-derived microorganisms. In a study by Valldecabres et al. (2022), cows supplemented with GF showed a positive effect on milk yield, with an increase of 2.96 kg/d, which is consistent with the milk yield improvement observed in our study. However, some studies have reported a lack of effect on milk production in mid-lactation cows (Dickerson et al., 2022; Goldsmith et al., 2023). Our study observed a clear separation between treatment and control groups after week 12 of lactation, which is similar to the results reported by Valldecabres et al. (2022). It is likely that a certain amount of time is needed for the supplemental strains to integrate with the pre-existing microbial population in the rumen and establish a new microbiome dynamic that can impact metabolic function and ultimately result in a measurable physiological shift in the cow (Valldecabres et al., 2022).

The effects of supplementing dairy cows with direct-fed microbials (DFM) on milk yield have been extensively studied, with varying results reported across studies. The inconsistencies in the results may be due to the multiple strains of fungi and bacteria found in commercially available supplements, as many of the commonly used strains are

not naturally present in the rumen (Ban & Guan, 2021). For example, while the fungal DFM Saccharomyces cerevisiae is one of the most used DFM products, some studies reported a lack of significant impact on milk production (AlZahal, McGill, et al., 2014; Ambriz-Vilchis et al., 2017; Bayat et al., 2015), while others reported an increase in milk yield (Desnoyers et al., 2009; Kumprechtová et al., 2019; Nocek & Kautz, 2006; Oh et al., 2019).

Another factor that has been studied in relation to DFM supplementation is milk composition. In our study, no significant differences were observed in milk fat and protein yield between GF and control groups, which is consistent with the results reported in some studies (Dickerson et al., 2022; Goetz et al., 2021; Goldsmith et al., 2023). However, Valldecabres et al. (2022) observed that GF supplementation tended to lower fat (%) in cows and reported a treatment x time effect on protein (%) with greater protein (%) in GF cows at week 11. The reasons for these outcomes remain unclear, but one potential explanation is the relationship suggested by Huws et al. (2018) between milk fat and protein contents and blood biomarkers NEFA and BHB, which are commonly considered energy status indicators in dairy cows. Nonetheless, additional research is necessary to elucidate the underlying reasons behind these findings.

Finally, the effects of DFM and rumen-derived microorganisms on milk yield and composition have been investigated in numerous studies, with varying results reported. While some studies have reported an increase in milk yield with DFM supplementation, others have reported no significant impact. Similarly, the effects of GF supplementation on milk production and composition have been inconsistent across studies. It is likely that the efficacy of DFM and rumen-derived microorganisms may depend on factors such as

the timing and duration of supplementation, the composition of the microbial community in the rumen, and the interaction between the supplemented microorganisms and the pre-existing microbial population. Further research is necessary to clarify these factors and to identify the optimal conditions for supplementing dairy cows with microorganisms to improve milk yield.

Rumen Fermentation Profile

Total VFA production was not affected by GF supplementation in the current study. The effects of DFM supplementation on rumen fermentation have been inconsistent. Desnoyers et al. (2009) found that the addition of yeast led to a significant increase in the concentration of total VFA, which is not consistent with the current findings. Similarly, Oh et al. (2019) reported positive effects on total VFA when dairy cows were supplemented with a S. cerevisiae based DFM, associated with increased milk production. Conversely, negative effects have been reported with lower overall rumen VFA in S. cerevisiae supplemented late lactation dairy cows (Thrune et al., 2009). Some studies reported no effect on total VFA in dairy cows supplemented with S. cerevisiae as DFM (Bayat et al., 2015; Desnoyers et al., 2009; Jiang et al., 2017; Philippeau et al., 2017), which is consistent with the current study.

Studies conducted thus far assessing the impact of a DFM composed of *Clostridium beijerinckii*, *Pichia kudriavzevii*, *Ruminococcus bovis,* and *Butyrivibrio fibrisolvens* have not reported any findings pertaining to its effects on rumen fermentation end products. However, from individual evaluation in vitro of the microorganisms it is known that the main VFA produced by strains of *C. beijerincki* and *B.fibrisolvens* is

butyrate, while *R. bovis* mayor major product is acetate (Emerson & Weimer, 2017; Gaffney et al., 2021; Gomez-Flores et al., 2017; Maia et al., 2010). In agreement with those studies, our results show that supplementing GF had a treatment effect on butyrate concentrations being greater in GF than CON cows. However, acetate tended to be lower in GF cows suggesting that *C. beijerincki* and *B.fibrisolvens* might have interacted better with endogenous gut microbiota and the host than *R. bovis* (Ban & Guan, 2021).

The transition period from late pregnancy to lactation in dairy cows is crucial for successful milk production, and involves acclimation to a lactation diet resulting in increased levels of ruminal butyrate and valerate, while other VFAs such as iso-butyrate and isovalerate remain unchanged. A study conducted by Weiss et al. (2008) at Ohio State University found that supplementing a DFM containing a strain of propionate producer (Propionibacterium sp.) during the transition period resulted in increased levels of rumen propionate and butyrate, and reduced acetate levels in treated cows compared to the control group, although the effects varied depending on the sampling day. Our findings support the potential for supplementing DFM during the transition period to increase butyrate levels and reduce acetate levels, which may have a positive impact on milk production and cow health.

GF cows showed a significant increase in butyrate concentration at 1 and 30 days in milk (DIM), which suggests potential regulatory and immunological functions in cattle (Mamuad et al., 2019). Butyrate may also have potential benefits for preventing cow mastitis by inhibiting the NLP3 signaling pathway and histone deacetylation (Jiang et al., 2020). NLRP3 inflammasome has been suggested as a potential biomarker for metabolic diseases in periparturient dairy cows (Castillo et al., 2019). However, a study by

Engelking et al. (2022) found that feeding butyrate to transition cows did not result in postpartum differences in serum inflammatory markers. The observed trend for increased valerate in GF cows may be due to changes in the metabolic activities of ruminal bacteria, such as lactate-utilizer M. elsdenii (Mamuad et al., 2019).

Ruminal Bacterial Abundance

Various factors, such as nutritional management and diet, can influence the composition of microbial populations in the rumen of ruminants (Bailoni et al., 2021). The composition of the rumen microbial population can shift during the transition period of dairy cows, as they are switched from a predominantly forage-based diet to a high-grain diet, which could lead to a reduction in fiber-digesting bacteria (Carpinelli et al., 2021; Clemmons et al., 2019; Minuti et al., 2015). The use of direct-fed microbials (DFM) can enhance gut health and animal performance, but the mechanisms through which they work are not yet fully understood, as they may vary depending on factors such as the type of microorganism used, the dose and duration of administration, and the characteristics of the host animal (Ban & Guan, 2021; McAllister et al., 2011). While bacterial and fungal DFM have been shown to affect ruminal lactic acid-utilizing bacteria (LUB) and lactic acid-producing bacteria (LAB), respectively, there is a lack of published research on the potential influence of rumen-derived supplements comprising yeast (P. kudriavzevii) and bacterial species (B. fibrisolvens, C. beijerinckii, and R. bovis) on the microbial population of dairy cows.

A study that reported that supplementation of live yeast in early lactation stimulated the lactate utilizing bacteria *M. elsdenii*, which helped counteract the effects of SARA (Pinloche et al., 2013). In agreement with this study, our results suggest that GF

supplementation tended to increase lactate digesting bacteria. In the present study lactate levels were not measured, however, we observed an increased abundance of *M. elsdenii* after parturition, which is in accordance with the notion of higher starch content in lactation diets. *M. elsdenii*, a potent lactate utilizer for the production of propionate and butyrate, has been found to be significantly enriched in efficient dairy cows (Shabat et al., 2016). The observed trend for increased *M. elsdenii* noted in GF cows may have contributed to the trend towards improved feed efficiency (milk production/DMI) during early lactation, as well as numerically greater feed efficiency throughout the trial period.

Similar to *M. elsdenii*, *A. lipolytica* can use lactate as a substrate for growth and has been associated with an increase in ruminal butyrate (Minuti et al., 2015). Increased abundance of A. lipolytica and B. fibrisolvens in transition dairy cows has been linked to starch digestibility associated with a high-carbohydrate diet postpartum (Edwards et al., 2017; Minuti et al., 2015; Vargas-Bello-Perez et al., 2016). These bacteria are also involved in the rumen biohydrogenation process in high-grain based lactating diets (Amin & Mao, 2021; Fuentes et al., 2009). Furthermore, live yeast supplementation has been reported to increase the ruminal abundance of A. lipolytica (AlZahal, Dionissopoulos, et al., 2014). Conversely, in this study *A. lipolytica* tended to be lower in GF cows at 7 and 100 DIM (Figure 2.4), suggesting that the treatment could have a direct or indirect impact on the growth or survival of this bacteria. The DFM supplemented in this study contained a strain of *B. fibrisolvens*, which could have competed with *A. lipolytica*, and, in turn, this could partially explain the trend for lower abundance of *A. lipolytica.*

Fibrobacter succinogenes has been recognized as a major ruminal cellulolytic bacteria involved in active hemicellulose hydrolysis and/or utilization (Emerson & Weimer, 2017; Jiang et al., 2017). *S. cerevisiae* supplementation in cows fed low-quality forage or under SARA conditions has been reported to increase the abundance of F. succinogenes (AlZahal, Dionissopoulos, et al., 2014; Amin & Mao, 2021; Malekkhahi et al., 2016). In contrast, other studies reported no effect of yeast supplementation on rumen bacterial abundance of *F. succinogenes* (Bayat et al., 2015; Silberberg et al., 2013). Minuti et al. (2015) reported that the abundance of *F. succinogenes* decreased in transition dairy cows after calving, which was mainly attributed to the high grain content in the lactation diet. A trend for lower *F. succinogenes* in GF compared to control was observed in this study, suggesting that the DFM failed to stimulate the growth of this fiber digesting bacteria. The observed trend for lower abundance of *F. succinogens* may have contributed to the trend for lower acetate proportion in GF cows. This is because these bacteria have been associated to an increase in ruminal acetate production (Malekkhahi et al., 2016).

Prevotella spp. abundance increases when transitioning from a high forage to a high concentrate diet (Bekele et al., 2010; Fernando et al., 2010; Petri et al., 2013), and an increase in P. bryantii postpartum has been observed (Minuti et al., 2015). In the current study, response due to GF supplementation was only observed for P. albensis with tendency to increase abundance in GF cows, suggesting that this bacteria could have been affected by GF supplementation beyond the expected increase due to the transition to a lactation diet (Carpinelli et al., 2021). Studies have reported that Prevotella spp. are the most abundant bacteria in the rumen, potentially due to their functional diversity and

nutritional versatility (Carpinelli et al., 2021; Clemmons et al., 2019; Plaizier et al., 2017). Prevotella spp. are able to degrade a range of substrates such as starch, hemicellulose, pectin, β-glucans, and proteins, to produce acetate, propionate, succinate, and formate (Carpinelli et al., 2021; David M. Stevenson & Paul J. Weimer, 2007). Although Prevotella spp. are the most abundant bacteria, the majority of Prevotella species in the rumen remain uncultured (David M. Stevenson & Paul J. Weimer, 2007).

Collectively, we observed that including GF in the diet promoted changes in the abundance of amylolytic, cellulolytic and lactate-utilizing bacteria in the rumen. These changes appear to have a positive effect on rumen fermentation, especially with regards to butyrate, which might have improved VFA absorption by the host through rumen epithelial tissue. It is conjectured to be beneficial during periods of rapid dietary shifts, like the transition period. The abrupt change from a high-fiber diet prepartum to a high-starch postpartum diet could lead to a decreased rumen pH, which can predispose cows to metabolic diseases such as SARA leading to a dysbiosis in the rumen.

Blood biomarkers

Energy metabolism

During the transition period, cows experience negative energy balance (NEB) due to insufficient energy intake to meet the high demands for milk synthesis, resulting in reduced plasma glucose concentrations (Halfen et al., 2021; Drackley, 1999; Ingvarsten & Anderson, 2000; Zarrin et al., 2017). To adapt to NEB, cows increase lipid mobilization, leading to increased lipolysis and higher plasma levels of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) (Contreras & Sordillo, 2011; Mezzetti

et al., 2019). High levels of NEFA and BHB have been associated with increased risk of metabolic and infectious diseases due to suppressed immune function and excessive inflammation (LeBlanc, 2020; Bertoni et al., 2008; Minuti et al., 2015)

Currently, there is a lack of understanding in literature that examines the potential influence of the microorganisms present in GF rumen-derived supplement on health performance in transition dairy cows. Goldsmith et al. (2023), however, observed no effect of GF supplementation on mid-lactation cows in glucose and NEFA concentrations, BHB was not measured. Inconsistent responses on blood glucose, BHB and NEFA have been reported when supplementing other bacterial and/or fungal DFM (AlZahal, McGill, et al., 2014; Kumprechtová et al., 2019; Oetzel et al., 2007).

In the present study, GF supplemented cows showed 3.93% lower blood glucose concentrations compared to control cows (4.24 mmol/L vs 4.41 mmol/L). Although no statistical differences in NEFA levels were observed, yet GF supplemented cows showed a 22% greater concentration of NEFA compared to control cows (0.20 mmol/L vs 0.16 mmol/L), while a treatment effect was noted for BHB being 27.9% greater in GF cows compared to control (0.49 mmol/L vs 0.37 mmol/L). A postpartal decrease observed in glucose reached nadir levels at 7 DIM, concomitantly, an increase in NEFA and BHB was observed with the peak at 7 DIM. Similar glucose, NEFA and BHB responses were observed in transition dairy cows diagnosed with subclinical ketosis at 7 DIM (Mezzetti et al., 2019). It is noteworthy that the postpartal decrease of glucose and increase of NEFA and BHB in this study remained at nonpathological levels since concentrations remained above threshold values for hypoglycemia and below risk levels reported

hyperketonemia (Dubuc & Buczinski, 2018; Mezzetti et al., 2019; Wankhade et al., 2017).

Increased rumen butyrate has been associated with increased blood BHB production when supplementing *M. elsdenii* in dairy calves; hence, higher butyrate production in the rumen in GF cows could have increased ketogenesis in the rumen, leading to greater plasma BHB (Muya et al., 2015). It should be noted, however, the difference in physiological state. Inconsistent responses of the contribution of butyrate to plasma BHB have been reported. A recent study supplementing butyrate in the transition period observed no treatment effect on plasma BHB concentration (Engelking et al., 2022), which contrasts studies where butyrate increased plasma BHB (Halfen et al., 2021; Izumi et al., 2019). However, the latter refers to butyrate supplementation in early and late lactating stages where fatty acid mobilization likely did not occur, and BHB production in the liver is reduced, increasing the relative contribution of supplemented butyrate to plasma BHB (Engelking et al., 2022). Taken together, effects of infused butyrate, ruminal butyrate or BHB on lipolysis during the transition period is still unclear which warrants further study.

Liver Function

During the transition period, dairy cows experience an inflammatory state triggered by the release of proinflammatory cytokines (Bertoni et al., 2008; Bionaz et al., 2007; Carpinelli et al., 2023), which can lead to an acute-phase response in the liver, with an increase in positive acute proteins (such as ceruloplasmin and haptoglobin) and a decrease in negative acute proteins (such as albumin and retinol binding proteins) (Bertoni et al., 2008; Bionaz et al., 2007). Paraoxonase (PON), an enzyme synthesized

almost exclusively by the liver involved in antioxidant activity, is considered a negative acute phase protein and may serve as an index of liver function in dairy cows (Bertoni et al., 2008; Bionaz et al., 2007; Mezzetti et al., 2020). Its activity can decrease during an inflammatory state, such as the transition period after parturition, potentially indicating liver damage (Bionaz et al., 2007; Wankhade et al., 2017).

In the current study GF cows showed lower PON levels compared to control (76.8 U/mL vs 87.78 U/mL). In our study, the decrease in PON around parturition, regardless to of treatment, reached nadir levels at 7 DIM (Figure 2.6) and which is in agreement with previous studies in transition dairy cows (Bionaz et al., 2007; Minuti et al., 2015). Activity of PON in pregnant, early lactating and late lactating dairy cows has been previously investigated (Turk et al., 2005; Turk et al., 2004). They suggested that a decrease in PON after parturition may be due to 1) liver damage or dysfunction caused by fat mobilization and triglyceride deposition in hepatocytes (Turk et al., 2004); 2) reduction in blood HDL cholesterol (Turk et al., 2005); 3)increase in oxidative stress(Turk et al., 2005; Turk et al., 2004); 4) or combination of these. Although this might suggest that liver damage in GF cows was more severe, it is well known that concentrations of PON in blood decrease after parturition (Bionaz et al., 2007; Minuti et al., 2015; Trevisi & Minuti, 2018). Overall, the concentrations of PON postpartum were within the typical physiological range expected for early postpartal cows without clinical disease (Trevisi & Minuti, 2018). To our knowledge, no study has reported effects of DFM on paraoxonase PON levels during the transition period.

Inflammation

Ceruloplasmin and haptoglobin are important acute-phase proteins (APPs) that have a protective role against pathogens and reflect the alteration of liver function induced by the acute phase response (APR) in dairy cows (Mezzetti et al., 2020). HP is suggested as the most promising biomarker of inflammation in dairy cows due to its longer plasma half-life relative to other +APPs and is effective in the diagnosis and prognosis of diseases (e.g. mastitis and endometritis) (Murata et al., 2004). Ceruloplasmin is less commonly used for diagnosis, but it can be used as an indicator of infection and is correlated with markers of oxidative stress, inflammation, and the innate immune system (Trevisi & Minuti, 2018). Increased levels of HP and ceruloplasmin have been reported around parturition in previous studies (Bertoni et al., 2008; Bionaz et al., 2007; Mezzetti et al., 2020; Trevisi et al., 2012).

In the current study, GF cows showed greater concentration of HP and ceruloplasmin when compared to control (0.22g/L vs 0.14 g/L; 3.27/L µmol/L vs 3.01 µmol/L, respectively). In contrast, some studies supplementing DFM containing different strains of microorganisms than GF (*S. cerevisiae* with *E. faecium*, *Bacillus pumillus* or *S. cerevisiae boulardii*) during transition period reported no treatment effect on HP, where ceruloplasmin was not measured. To our knowledge, there are no studies reporting DFM supplementation effects on ceruloplasmin biomarker. The increase of both +APP around parturition, regardless to treatment, reached peak levels at 7 DIM (Figure 2.6 which panel of Figure), which agrees with previous studies (Bertoni et al., 2008; Trevisi et al., 2012). It is noteworthy to mention that although ceruloplasmin concentrations were above the suggested risk threshold level (> 2.7 µmol/L) (Trevisi & Minuti, 2018). However, HP

concentrations were below the values reported for healthy cows (Bertoni et al., 2008; Bionaz et al., 2007; Huzzey et al., 2009; Trevisi et al., 2012). Even though sudden changes in body homeostasis are to some degree considered normal during the peripartum period, the precise cause of immune dysfunction in cows has yet to be clearly established (Mezzetti et al., 2020).

Genes in polymorphonuclear leukocyte expression related to inflammation (TNF-α, IL-6), anti-inflammation (IL-10), and cell membrane receptors (SELL) changes in blood have been suggested to be associated with systemic inflammation and immune function conditions (Carpinelli et al., 2023; Sun et al., 2021). In a recent study, Hiltz et al. (2023) reported that supplementation of *S. cerevisiae boulardii* to transition dairy cows increased Tumor necrosis factor-α and tended to increase Interleukin-6, though no changes were seen in HP. They suggested that DFM supplementation may have caused an inconsistent acute immune response that did not affect all proinflammatory pathways. There is a lack of data regarding the influences of DFM on immune responses and inflammation in transition dairy cows, and further research is needed.

Oxidative stress

Increased levels of ROM around calving have been reported by several studies (Bionaz et al., 2007; Mezzetti et al., 2019; Trevisi et al., 2009). In our study, GF cows had greater ROM concentration when compared to control cows. Concentration of ROM peaked at 7 DIM regardless of treatment group, which is in agreement with findings observed previously by Bionaz et al. (2007) and Mezzetti et al. (2019). An increase in ROM can cause detrimental effects if the antioxidant capacity is overwhelmed (Abuelo et al., 2015). Ferric reducing ability plasma (FRAP) was the only antioxidant capacity

biomarker measured in this study which did not vary between treatment and control group. A standardization of oxidative stress status in dairy cows is yet to be established (Abuelo et al., 2015); determining threshold values because a proper evaluation of oxidative status for preventing and, ultimately, treating the effects of oxidative stress is fundamental in ruminant medicine (Celi, 2011).

Normal parturition in dairy cows can cause inflammation, leading to oxidative stress due to an imbalance between reactive oxygen metabolites (ROM) production and the antioxidant mechanisms' neutralizing capacity (Bionaz et al., 2007). Oxidative stress can modify physiological and metabolic functions, leading to pathologies (Bernabucci et al., 2005). Elevated ROM concentrations indicate stronger inflammation, leading to a higher susceptibility to diseases (Trevisi et al., 2015). An increase in production of NEFA leads to a corresponding increase in ROM production due to beta-oxidation in peroxisome (Mezzetti et al., 2020).

Turk et al. (2005) suggests that decreased levels of PON may cause a decline in antioxidative protection during early lactation. PON inversely correlates with oxidative stress as it has the ability to protect low-density and high-density lipids from lipid peroxidation. An increase in ROM coupled with a decreased PON in GF than CON cows indicates that oxidative stress was probably caused by a benign inflammatory condition produced by GF supplementation (Mezzetti et al., 2020). It is suggested that some degree of inflammation after calving is not a pathological process, but rather an adaptive process that is necessary for a successful transition period adaptation and milk production (Abuelo et al., 2015). Further research is needed to clearly elucidate the effects of DFM

on the redox balance in dairy cows as no studies have reported DFM supplementation effects on oxidative stress.

CONCLUSIONS

Direct-fed microbial have received greater attention in the last decade as a solution to improve production efficiency in ruminants. However, data of rumen derived DFM supplementation in dairy cows is scarce. The findings of this study revealed that supplementation of a rumen derived DFM comprised of Clostridium beijerinckii, Pichia kudriavzevii, Ruminococcus bovis, and Butyrivibrio fibrisolvens (GF), promoted positive responses in performance, such as milk yield and feed efficiency in terms of milk/DMI. In case of rumen VFA, GF contributed to increase butyrate and valerate after calving. The latter was accompanied by increment in lactic utilizing bacterial abundance in the rumen microbiota. Moreover, GF supplementation may have influenced lipid metabolism, leading to greater oxidative stress and inflammation state within non-pathological levels. Nonetheless, the effects of DFM on health status in transition dairy cows and its mechanisms remain to be elucidated. In conclusions, our findings indicated that supplementing GF during the transition period through mid-lactation has a positive impact on both rumen environment and production performances, suggesting an overall beneficial effect.

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Table 2.1 - Ingredient composition of diets during the close-up (-21d) and lactation periods.

	Diet	
Component	Close-up	Lactation
Ingredient, % DM[1]		
Corn silage	40.22	34.56
Alfalfa hay	----	14.58
Grass Hay	21.68	----
Cotton seed	----	7.20
Wheat straw	10.14	----
Molasses	----	3.70
Dry cow grain mix[2]	27.96	----
Lactating cow grain mix[3]	----	36.29
Chemical analysis		
DM, %	49.81	50.61
NE_L, Mcal/ kg DM	1.56	1.76
CP, % DM	14.46	15.69
NDF, % DM	43.73	31.37
ADF, % DM	29.04	20.52

[1]Ingredients included in the ration formulated using AMTS

[2]Dry cow grain mix contained (as % DM): Soybean meal 47.5 solvent (44.6), distillers grain dry (13.1), soy hulls (11.5), limestone Ca (8.4), biochlor (6.8), corn grain ground fine (4.8), magnesium sulfate 7H2O (2.1), reashure choline (1.6), calcium chloride (1.4), magnesium oxide (1.4), vitamin E (0.9), calcium sulfate dehydrate (0.9), JPW dairy vitamin premix (0.6), calcium phosphate monocal (0.6), Chromium 4% premix (0.5), salt white (0.4), JPW dairy TM premix (0.4).

[3]Lactating cow grain mix contained (as % DM): corn grain ground fine (49.8), soybean meal (19.9), soy best (14.0), distillers grain dry (4.4), sodium bicarbonate (3.0), limestone Ca (2.7), energy booster 100 (1.9), salt white (1.0), urea (0.9), soy hulls (0.6), magnesium oxide (0.6), calcium phosphate monocal (0.6), JPW dairy TM premix (0.2), JPW dairy vitamin premix (0.2), vitamin E (0.1), Biotin 2% (0.005).

Table 2.2 - Species-specific primers used in real-time qPCR assay for the quantification of selected rumen bacteria population.

Target bacterial species		Primer sequence (5'- 3')	Reference
Anaerovibrio lipolytica	F[a]	GAAATGGATTCTAGTGGCAAACG	(Abdelmegeid et al., 2018)
	R[b]	ACATCGGTCATGCGACCAA	
Butyrivibrio fibrisolvens	F	ACACACCGCCCGTACCA	(Klieve et al., 2003)
	R	TCCTTACGGTTGGGTCACAGA	
Butyvibrio proteoclasticus	F	GGGCTTGCTTTGGAAACTGTT	(Abdelmegeid et al., 2018)
	R	CCCACCGATGTTCCTCCTAA	
Eubacterium ruminantium	F	CTCCCGAGACTGAGGAAGCTTG	(Abdelmegeid et al., 2018)
	R	GTCCATCTCACACCACCGGA	
Fibrobacter succinogenes	F	GCGGGTAGCAAACAGGATTAGA	(Abdelmegeid et al., 2018)
	R	CCCCCGGACACCCAGTAT	
Megaspheara elsdenii	F	AGATGGGGACAACAGCTGGA	(Abdelmegeid et al., 2018)
	R	CGAAAGCTCCGAAGAGCCT	
Prevotella albensis	F	GCGCCACTGACGCTGAAG	(Khafipour et al., 2009)
	R	CCCCAAATCCAAAAGGACTCAG	
Prevotella brevis	F	GGTTTCCTTGAGTGTATTCGACGTC	(Stevenson & Weimer,
	R	CTTTCGCTTGGCCGCTG	2007)
Prevotella bryantii	F	AGCGCAGGCCGTTTGG	(Abdelmegeid et al., 2018)
	R	GCTTCCTGTGCACTCAAGTCTGAC	
Prevotella ruminicola	F	GAAAGTCGGATTAATGCTCTATGTTG	(Stevenson & Weimer,
	R	CATCCTATAGCGGTAAACCTTTGG	2007)
Rumicoccus albus	F	ACGTCRTCCMCACCTTCCTC	(Koike & Kobayashi, 2001)
	R	CCTCCTTGCGGTTAGAACA	
Rumicoccus flavefaciens	F	CGAACGGAGATAATTTGAGTTTACTTAGG	(Denman & McSweeney,
	R	CGGTCTCTGTATGTTATGAGGTATTACC	2006)
Ruminobacter amylophilus	F	CTGGGGAGCTGCCTGAATG	(Stevenson & Weimer, 2007)
	R	GCATCTGAATGCGACTGGTTG	
Selenomonas ruminantium	F	CAATAAGCATTCCGCCTGGG	(Abdelmegeid et al., 2018)
	R	TTCACTCAATGTCAAGCCCTGG	
Succinimonas amylolytica	F	CGTTGGGCGGTCATTTGAAAC	(Abdelmegeid et al., 2018)
	R	CCTGAGCGTCAGTTACTATCCAGA	
Succinivibrio dextrinosolvens	F	TAGGAGCTTGTGCGATAGTATGG	(Abdelmegeid et al., 2018)
	R	CTCACTATGTCAAGGTCAGGTAAGG	
Streptococcus bovis	F	TTCCTAGAGATAGGAAGTTTCTTCGG	(Abdelmegeid et al., 2018)
	R	ATGATGGCAACTAACAATAGGGGT	
Treponema bryantii	F	AGTCGAGCGGTAAGATTG	(Tajima et al., 2001)
	R	CAAAGCGTTTCTCTCACT	
Bacteria general 1	F	GGATTAGATACCCTGGTAGT	(Abdelmegeid et al., 2018)
	R	CACGACACGAGCTGACG	
Bacteria general 2	F	GTGSTGCAYGGYTGTCGTCA	(Abdelmegeid et al., 2018)
	R	ACGTCRTCCMCACCTTCCTC	

[a]F: forward primer

[b]R: reverse primer

Table 2.3 - Body weight, BCS, DMI, and energy balance of dairy cows during the peripartal period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial.

	Treatment			P-value		
Parameter	**GF**	**CON**	**SEM[3]**	**Trt**	**Time**	**Trt x Time[4]**
Prepartum[1]						
BW, kg	715.09	712.8	16.38	0.88	<0.01	0.50
BCS	3.46	3.4	0.03	0.17	0.66	0.71
DMI, kg/day	12.69	13.62	0.55	0.19	0.12	0.07
Energy balance, Mcal/d	-1.6	-1.8	1.34	0.82	0.01	0.63
Postpartum[2]						
BW, kg	633.75	637.97	12.97	0.73	<0.01	0.07
BCS	2.93	2.97	0.04	0.29	<0.01	0.93
DMI, kg/day	20.79	20.34	0.44	0.43	<0.01	0.25
Energy balance, Mcal/d	-0.96	-1.08	0.54	0.86	<0.01	0.08

[1]Prepartum parameters were analyzed from -21 d to calving.
[2]Postpartum parameters were analyzed from calving to 100 DIM.
[3]Largest standard error of the mean.
[4]Interaction of treatment and weeks in milk.

Table 2.4 - Milk production and composition of dairy cows during the peripartal period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial.

	Treatment			P-value		
Item	**GF**	**CON**	**SEM[1]**	**Trt**	**Time**	**Trt x Time[2]**
Milk yield, kg/d	37.60	35.95	0.86	0.13	<0.01	0.47
Early lactation[4]	33.82	32.97	0.84	0.43	<0.01	0.47
Mid lactation[5]	39.71	37.07	1.06	0.07	<0.01	0.24
Milk composition						
Fat %	3.23	3.36	0.07	0.18	<0.01	<0.01
Protein %	3.01	3.08	0.04	0.17	<0.01	<0.01
SCC[3]	1.72	1.52	0.29	0.47	<0.01	0.89
MUN	9.71	9.42	0.37	0.43	<0.01	0.27
Yield of milk components						
Milk fat yield, kg/d	1.19	1.17	0.04	0.58	0.06	0.16
Milk protein yield, kg/d	1.15	1.12	0.03	0.39	0.63	0.46
ECM, kg/d	36.74	35.46	0.90	0.19	0.04	0.51
Feed Efficiency, kg/kg	0.87	0.80	0.03	0.16	<0.01	0.26
Early lactation	0.96	0.88	0.03	0.10	0.68	0.23
Mid lactation	0.82	0.76	0.03	0.26	<0.01	0.21

[1]Largest standard error of the mean
[2]Interaction of treatment and weeks in milk
[3]Somatic cell counts were transformed to Log10.
[4]Early lactation corresponds to weeks 1 to 4 after parturition.
[5]Mid lactation corresponds to weeks 5 to 14 after parturition.

Table 2.5 - Ruminal VFA of dairy cows during the peripartal period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial.

	Treatment		SEM[1]	P-value		
Parameter	**GF**	**CON**		**Trt**	**Time**	**Trt x Time[2]**
pH	6.46	6.51	0.05	0.43	<0.01	0.82
NH_3, mg/dL	10.23	10.43	0.57	0.79	<0.01	0.69
Total VFA, nM	113.01	113.80	3.32	0.87	0.04	0.65
VFA mol/100 mol						
Acetic acid	71.18	72.32	0.55	0.10	<0.01	0.77
Propionic acid	17.41	16.98	0.54	0.47	<0.01	0.69
Butyric acid	7.89	7.4	0.19	0.04	0.01	0.10
Valeric acid	1.29	1.19	0.04	0.06	0.05	0.64
Caproic acid	0.63	0.61	0.02	0.52	<0.01	0.09
Iso-butyric acid	0.86	0.87	0.02	0.71	<0.01	0.81
Iso-valeric acid	0.71	0.72	0.02	0.73	<0.01	0.48
Acetate: Propionate	4.43	4.53	0.17	0.60		0.59

[1]Largest standard error of the mean.
[2]Interaction of treatment and days in milk.

Table 2.6 - Relative abundance (%) of target bacterial species mixed ruminal fluid from peripartal dairy cows fed basal diet without (CON) or with (GF) direct-fed microbial.

	Treatment			P-value		
Species[2]	**GF**	**CON**	**SEM[1]**	**Trt**	**Time**	**Trt x Time[3]**
Anaerovibrio lipolytica	2.17×10^{-02}	2.71×10^{-02}	0.21	0.27	<0.01	0.08
Butyrivibrio fibrosolvens	3.91×10^{-03}	3.83×10^{-03}	0.16	0.91	0.03	0.40
Butyrivibrio proteoclasticus	2.00×10^{-01}	2.41×10^{-01}	0.23	0.27	<0.01	0.65
Eubacterium ruminatium	7.34×10^{-02}	6.25×10^{-02}	0.14	0.19	0.72	0.90
Fibrobacter succinogens	1.97×10^{-01}	2.92×10^{-01}	0.22	0.07	0.02	0.39
Megasphaera elsdenii	7.35×10^{-03}	4.61×10^{-03}	0.26	0.07	<0.01	0.85
Prevotella albensis	2.37×10^{-02}	1.11×10^{-02}	0.42	0.09	<0.01	0.42
Prevotella bryantii	1.75×10^{-01}	1.09×10^{-01}	0.38	0.15	<0.01	0.38
Prevotella ruminicola	$1.70 \times 10^{+00}$	$2.07 \times 10^{+00}$	0.12	0.12	0.06	0.72
Prevotella brevis	1.45×10^{-01}	1.29×10^{-01}	0.09	0.23	0.27	0.18
Ruminococcus albus	1.23×10^{-06}	1.52×10^{-06}	0.34	0.43	0.69	0.41
Ruminococcus flavefaciens	3.13×10^{-02}	4.10×10^{-02}	0.28	0.31	0.03	0.86
Ruminobacter amylophilus	2.91×10^{-02}	3.81×10^{-02}	0.78	0.59	<0.01	0.77
Selenomonas ruminatium	$1.50 \times 10^{+00}$	$1.64 \times 10^{+00}$	0.13	0.50	<0.01	0.75
Succinimonas amylolytica	1.05×10^{-02}	8.69×10^{-03}	0.70	0.78	<0.01	0.21
Succinivibrio dextrinosolvens	2.37×10^{-02}	3.48×10^{-02}	0.62	0.35	<0.01	0.31
Streptococcus bovis	3.09×10^{-03}	2.62×10^{-03}	0.18	0.35	<0.01	0.93
Treponema byrantii	3.68×10^{-04}	2.45×10^{-04}	0.64	0.33	0.53	0.41

[1]Largest standard error of the mean is shown.
[2]Data were log-transformed before statistics. The standard errors of the means associated with log-transformed data are in log scale.
[3]Interaction of treatment and days in milk.

Table 2.7 - Blood biomarkers related to energy and nitrogen metabolism, liver function, inflammation and oxidative stress of dairy cows during the peripartal period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial.

	Treatment			P-value			
Parameter	**GF**	**CON**	**SEM[1]**	**Trt**	**Parity**	**Time**	**Trt x Time[3]**
Energy Metabolism							
Glucose, mmol/L	4.24	4.41	0.06	0.03	0.05	<0.01	0.74
NEFA, mmol/L[2]	0.20	0.16	0.17	0.23	-	<0.01	0.82
BHB, mmol/L[2]	0.49	0.37	0.14	0.03	0.18	<0.01	0.80
Nitrogen Metabolism							
Urea, mmol/L	4.55	4.54	0.15	0.93	-	<0.01	0.36
Protein, g/L	73.62	73.54	0.72	0.93	-	<0.01	0.74
Creatinine, µmol/L	88.43	88.42	1.13	1.00	-	<0.01	0.74
Liver Function							
Total Bilirrubin, µmol/L	2.60	2.49	0.15	0.57	-	<0.01	0.96
GOT[4], U/L	103.21	93.65	4.89	0.11	0.72	<0.01	0.20
GGT[5], U/L	19.34	20.51	0.88	0.33	-	<0.01	0.89
Cholesterol, mmol/L	2.97	3.02	0.10	0.61	0.03	<0.01	0.91
Paraoxonase, U/mL	76.80	87.78	4.00	0.03	0.83	<0.01	0.65
Albumin, g/L	33.62	33.81	0.39	0.70	-	<0.01	0.38
Inflammation and acute phase proteins							
Ceruloplasmin, µmol/L	3.27	3.01	0.08	0.01	0.05	<0.01	0.68
Haptoglobin, g/L	0.22	0.14	0.21	0.02	-	<0.01	0.74
Globulin, g/L	40.80	40.19	0.74	0.45	0.21	<0.01	0.94
Oxidative Stress							
FRAP[6], µmol/L	135.48	136.51	2.27	0.74	-	<0.01	0.13
ROM[7], mg of H2O2/100 mL	17.43	15.65	0.47	0.01	-	<0.01	0.99

[1]Largest standard error of the mean is shown.
[2]Data were log-transformed before statistics. The standard errors of the means associated with log-transformed data are in log scale.
[3]Interaction of treatment and days in milk.
[4]Glutamate oxaloacetate transaminase.
[5]Gamma-glutamyl transferase.
[6]Ferric-reducing antioxidant power.
[7]Reactive oxygen metabolites.

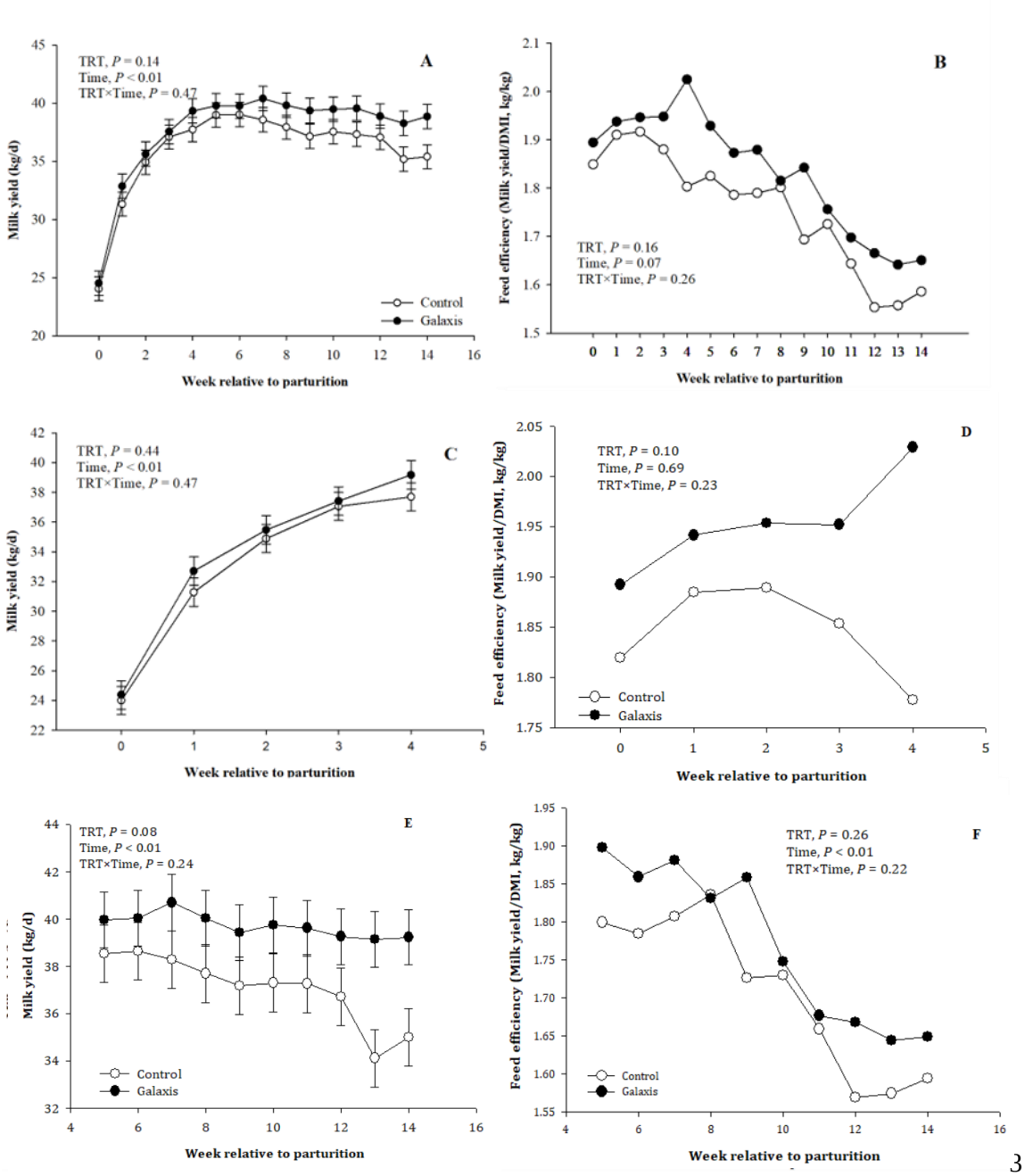

Figure 2.1 - Milk yield (A), Milk yield/DMI (B), Early-lactation milk yield (C), Early-lactation milk yield/DMI (D), Mid-lactation milk yield (E), and Mid lactation milk yield/DMI in cows during the transition period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial. Values are means and the standard errors are represented by vertical bars.

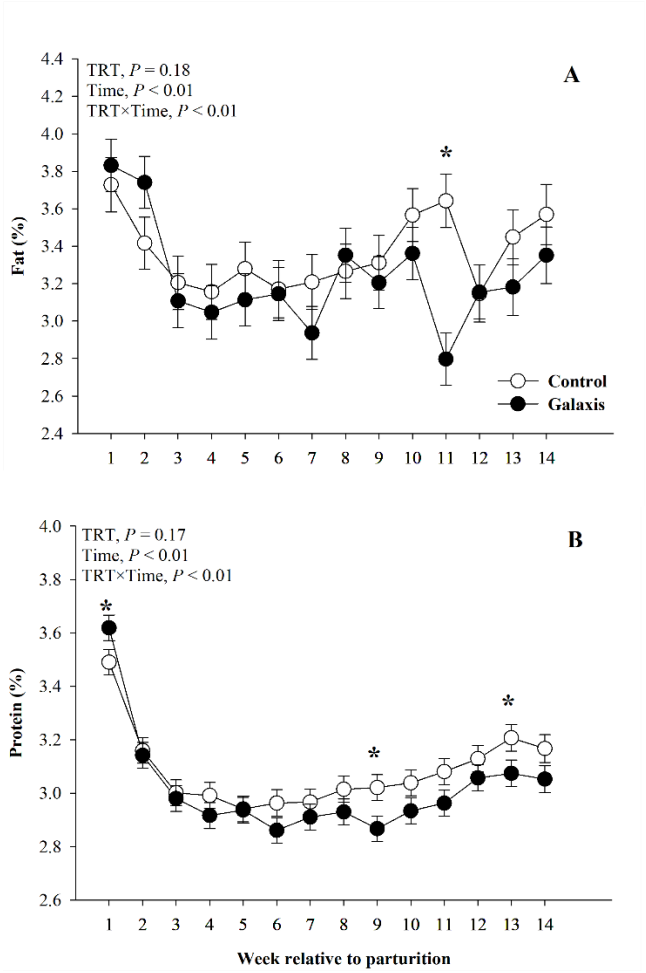

Figure 2.2 - Milk fat % (A) and protein % (B) in cows during the transition period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial. Values are means and the standard errors are represented by vertical bars.

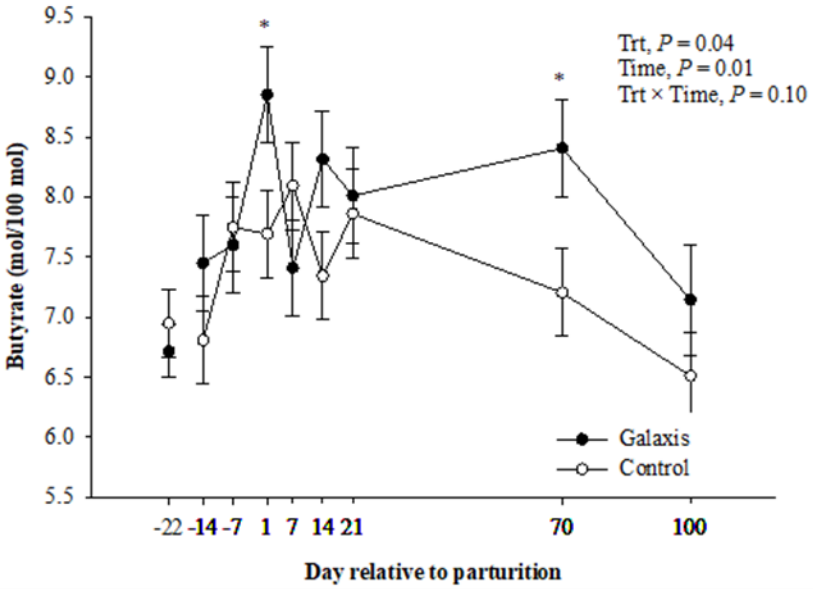

Figure 2.3 - Ruminal butyrate in dairy cows during the transition period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial. Values are means and the standard errors are represented by vertical bars.

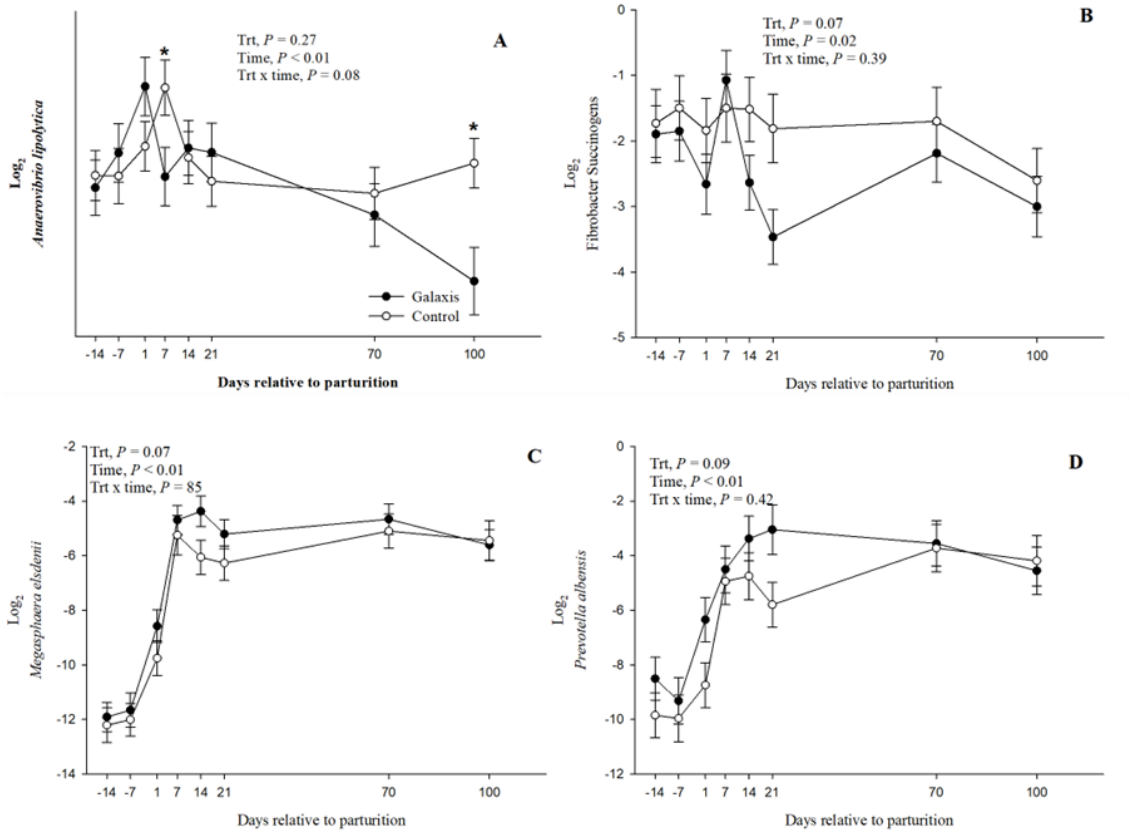

Figure 2.4 - Relative abundance (%) of microbial species in rumen fluid in dairy cows during the peripartal period until 100 DIM fed basal diet without (CON) or with (GF) direct-fed microbial. Values are means and the standard errors are represented by vertical bars.

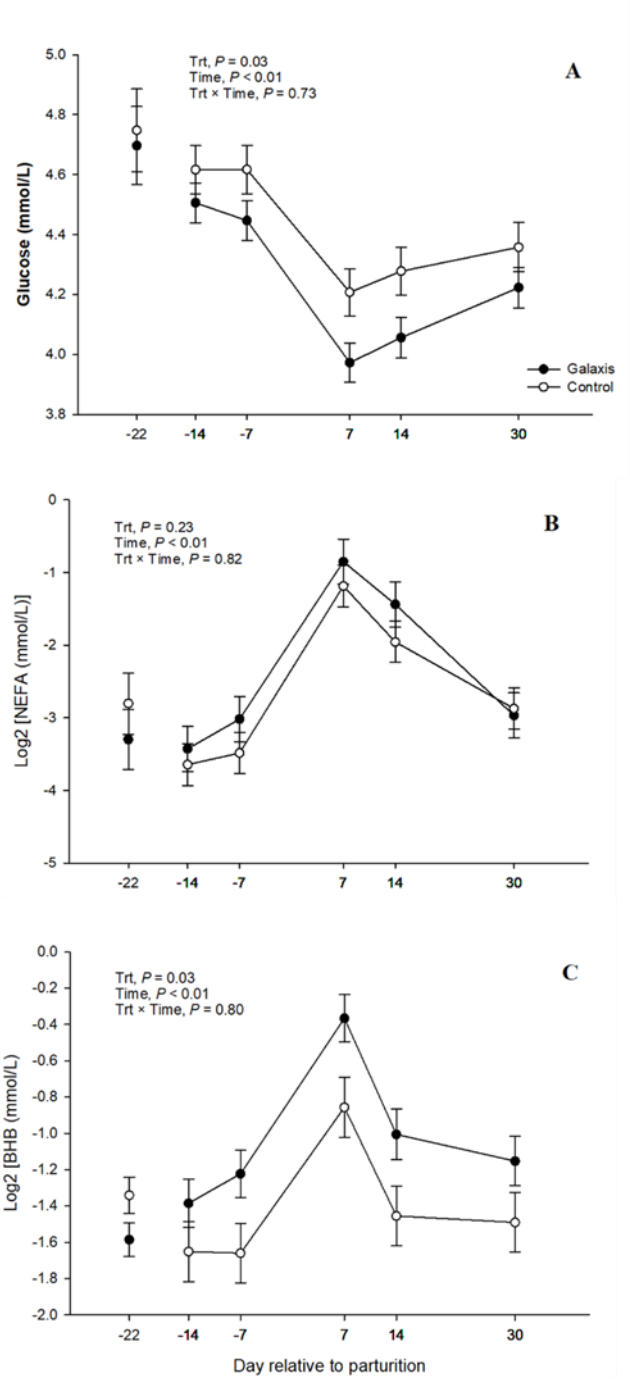

Figure 2.5 - Blood Glucose (A), NEFA (B) and BHB (C) in dairy cows during the transition period until 100DIM fed basal diet without (CON) or with (GF) direct-fed microbial. Values are means and the standard errors are represented by vertical bars.

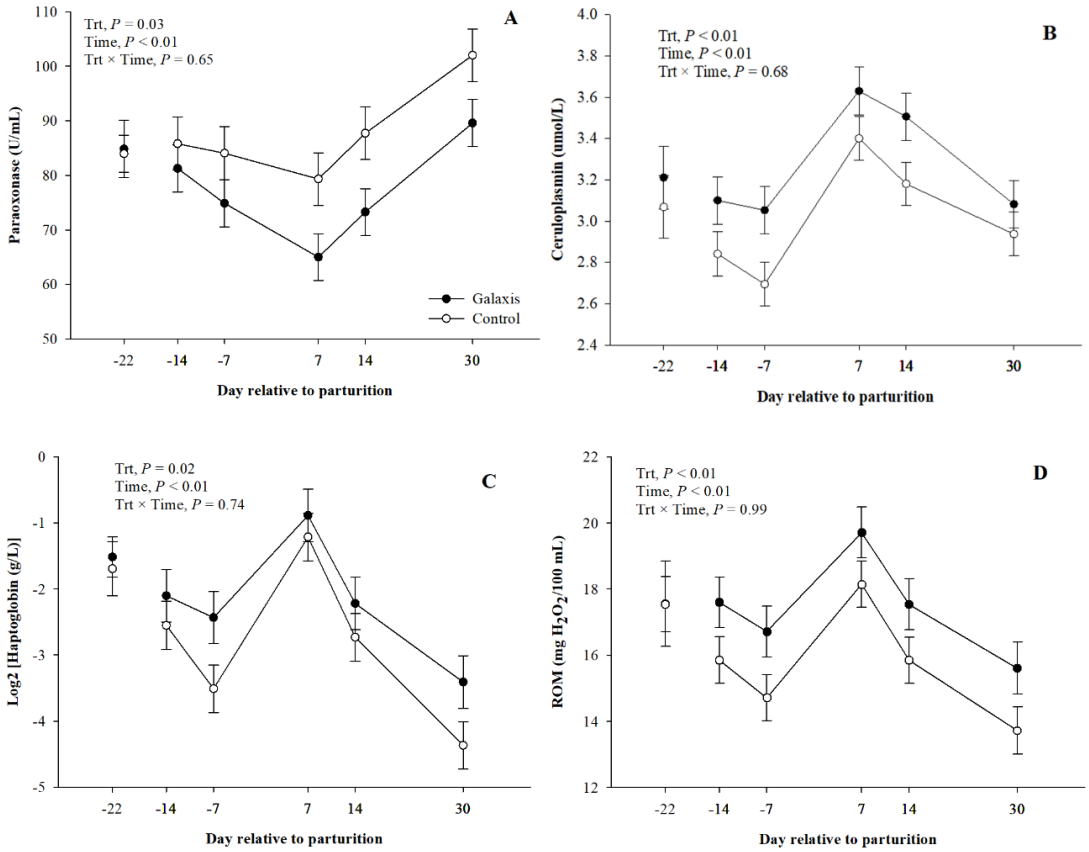

Figure 2.5. Blood Paraoxonase (A), Ceruloplasmin (B), Haptoglobin (C), and Reactive-oxygen metabolites (D) in dairy cows during the transition period until 100DIM fed basal diet without (CON) or with (GF) direct-fed microbial. Values are means and the standard errors are represented by vertical bars.

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