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Effects of peripartal rumen-derived direct-fed microbial supplementation on lactation performance, metabolism, ruminal fermentation, and microbial abundance in dairy cows

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ABSTRACT

The objective of this study was to evaluate the effects of a rumen-derived direct-fed microbial (DFM) product on performance, blood biomarkers, ruminal fermentation, and bacterial abundance in dairy cows during the transition period until 100 DIM. Fifty-six Holstein cows were enrolled in a randomized complete block design from −21 to 100 DIM. Cows were blocked based on expected calving date, parity, and previous lactation milk yield for multiparous cows or genetic merit for primiparous cows. At −21 DIM, cows were randomly assigned to either a basal control diet supplemented with 150 g/d ground corn (CON; n = 29) or the control diet supplemented with ground corn plus a rumen-derived DFM product (GF; n = 27, 150 g/d ground corn + 5g/d of Galaxis Frontier [Native Microbials, San Diego, CA]; *Clostridium beijerinckii* at 1.0×10^7 cfu; *Pichia kudriavzevii* at 1.0×10^8 cfu; *Ruminococcus bovis* at 1.0×10^8 cfu; *Butyrivibrio fibrisolvens* at 1.0×10^8 cfu) that was top-dressed once a day. All cows received the same basal close-up diet from −21 DIM until calving (1.56 Mcal/kg DM and 14.46% CP) and the same lactation diet from calving to 100 DIM (1.76 Mcal/kg DM and 15.69% CP). We collected blood samples to measure biomarkers of metabolism, inflammation, and oxidative stress, as well as rumen fluid via esophageal tubing for ammonia, VFA, and microbial abundance from a subset of multiparous cows (n = 12/treatment) at various time points from −22 to 100 DIM. Compared with CON, GF cows produced more milk (+4.1 kg/d) during the postfresh period (6–14 wk). However, GF cows tended to produce more milk (+2.9 kg/d) than CON during the entire trial (0–14 wk). Although DMI was not affected by treatment, GF cows had greater feed efficiency (+0.18, milk/DMI) in the postfresh period. Compared with CON, GF cows had lower blood plasma glucose and higher BHB. Blood biomarkers showed greater concentrations of ceruloplasmin, haptoglobin, and reactive oxygen metabolites (ROM) in GF cows compared with CON. Compared with CON, GF cows had greater ruminal molar proportions of butyrate and tended to have greater valerate and lower acetate. These changes in ruminal VFA were coupled with alterations in ruminal microbial abundance, where compared with CON, GF cows tended to have a greater abundance of lactate-utilizing species (*Megasphaera elsdenii*), but lower abundance of cellulose-utilizing species (*Fibrobacter succinogenes*). Although greater ROM was accompanied by a mild inflammatory condition in GF cows, this was not detrimental to milk yield and DMI. Overall, our results suggest that supplementing GF in the transition period until 100 DIM positively affects lactation performance.

Key words: direct-fed microbials, transition cow, ruminal microbial abundance, feed additive

INTRODUCTION

During the transition period, defined as 3 wk before parturition to 3 wk after parturition, cows undergo drastic adaptive physiological, metabolic, and immunological changes (Drackley, 1999). Negative energy balance (**NEB**), resulting from an inadequate energy intake in view of the high demand for milk synthesis, usually leads to BCS loss, a high risk of metabolic disorders, and alterations in immune function (Ingvartsen and Moyes,

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2013). The immune dysfunction and metabolic stress increase the likelihood of developing health disorders during early lactation (Sordillo and Aitken, 2009).

Direct-fed microbials (**DFM**) are feed additives commonly used to improve production, efficiency and health in dairy cows (Yoon and Stern, 1995). We use the term direct-fed microbials as defined previously, as "live, naturally occurring microorganisms that have been used to improve digestive function of livestock" (Yang et al., 2004, p. 180). Direct-fed microbials, by this definition, can be classified into 3 categories: bacterial, fungal, or a combination of both (Elghandour et al., 2015). Several benefits and modes of action have been proposed for DFM, including control of rumen pH driven by stimulation of lactic acid–utilizing bacteria, enhancement of rumen native microbiota by provision of growth factors, and improved nutrient uptake by increasing substrate breakdown (Nocek et al., 2003; Nocek and Kautz, 2006; Ban and 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 (e.g., bacterial or fungal), the dose and duration of administration, and the characteristics of the host animal and its microbiome (Ban and Guan, 2021).

Several studies have reported positive effects of supplementing conventional DFM on the performance of dairy cows in the transition period, as well as in early lactation and mid-lactation (Nocek and Kautz, 2006; Oh et al., 2019; Hiltz et al., 2023). Moreover, previous studies have investigated the influence of these microbial additives on ruminal fermentation (Chiquette et al., 2015; Philippeau et al., 2017; Mamuad et al., 2019), energy metabolism (Oetzel et al., 2007; AlZahal et al., 2014; Luan et al., 2015), and inflammatory response (Hiltz et al., 2023). Common bacteria and fungi used in conventional DFM are not native to the ruminal environment (Kamra, 2005); therefore, they may have a limited ability to manipulate and interact with the rumen and its native microbial community (Moraïs and Mizrahi, 2019).

Current research on rumen-derived DFM has evaluated parameters related to lactation performance and feed efficiency in early lactation (Valldecabres et al., 2022; Nehme Marinho et al., 2024) and mid-lactation cows (Goetz et al., 2021; Dickerson et al., 2022; Goldsmith et al., 2023). However, further exploration of how rumen-derived DFM affect ruminal fermentation profile is warranted. Additionally, Goldsmith et al. (2023) observed a trend for lower insulin when cows were supplemented with rumen-derived DFM, which was associated with lower DMI and, consequently, lower ruminal propionate. These effects are particularly important in the context of transition dairy cows, when low DMI, ruminal propionate, and insulin are commonly observed around parturition. It is therefore relevant to determine if these effects may improve lactation performance, feed efficiency, or health outcomes during the transition period. In this study, we evaluated a rumen-derived DFM composed of 3 bacterial species (*Clostridium beijerinckii*, *Ruminococcus bovis*, and *Butyrivibrio fibrisolvens*) and 1 fungal species (*Pichia kudriavzevii*) in transition dairy cows. The rumen-derived DFM were all originally isolated from the rumen of high-performing dairy cows (Zengler and Embree, 2016). *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 (Fernandes et al., 2019; Suntara et al., 2021). Conversely, *C. beijerinckii* is not able to degrade cellulose; however, it has been reported to produce butyrate and acetate when cocultured with other complex carbohydrate degraders (Gomez-Flores et al., 2017). Additionally, *B. fibrisolvens* is a well-known bacterium and one of the primary species responsible for the biohydrogenation process in the rumen (Amin and Mao, 2021). *Ruminococcus bovis,* a recently isolated species, is able to degrade resistant starch with acetate as the major fermentation product (Gaffney et al., 2021).

Currently, limited data are available on the effects of rumen-derived DFM used in this study on ruminal fermentation profiles. Rumen-derived DFM have been observed to improve feed efficiency in early lactation cows (Valldecabres et al., 2022) and to a lesser extent in mid-lactation cows (Dickerson et al., 2022; Goldsmith et al., 2023). Therefore, we hypothesized that supplementing rumen-derived DFM would improve forage digestibility and ruminal VFA production, and consequently promote increased milk production and feed efficiency. The objective of this study was to evaluate the effects of a rumen-derived DFM on performance, blood biomarkers, ruminal fermentation, and bacterial abundance in dairy cows during the transition period until 100 DIM.

MATERIALS AND METHODS

Experimental Design, Dietary Treatments, and Sample Size Calculation

All the procedures for this study were approved by the Institutional Animal Care and Use Committee of South Dakota State University (Brookings, SD; protocol no. 2011-053A). Seventy-two Holstein cows, including 57 multiparous and 15 primiparous were enrolled in a randomized complete block design, where expected calving date, parity, and previous lactation milk yield for multiparous or genetic merit for primiparous cows were used as blocking factors. A total of 16 cows were removed from the experiment due to acute health conditions lead-

Table 1. Frequency of occurrence of health problems in peripartal dairy cows fed a control diet (CON) or control diet plus direct-fed microbial (GF) from −21 ± 5 d relative to calving through 100 DIM

	Treatment	
Event	CON	GF
Total	29	27
Ketosis[1]	3	7
Lameness[2]	1	0
Mastitis[3]	7	2
Metritis[4]	6	6
Retained placenta[5]	3	2
Cows with >1 disease	7	2

[1]Defined as cows having (>1.4 mmol/L) ketone concentration in blood, detected using a Precision Xtra (Abbott Laboratories, Abbott Park, IL). The treatment administered was oral propylene glycol and injection of the vitamin B complex.

[2]Defined as cows having locomotion score (>1).

[3]Determined based on the California Mastitis Test.

[4]Foul smelling, watery consistency uterine discharge after calving.

[5]Defined as fetal membranes retained >24 h postpartum.

ing to low intake (n = 7), as well as twins (n = 2), calving with <14 d on treatment diets (n = 2), being unadapted to the feeding gate system (n = 3), abortion (n = 1), and death (n = 1; Supplemental Table S1, see Notes). Data from 56 cows (43 multiparous and 13 primiparous) were used in the analyses. Health conditions observed among these 56 cows included ketosis (n = 10; 8 multiparous and 2 primiparous), lameness (n = 1 multiparous), mastitis (n = 9; 8 multiparous and 1 primiparous), metritis (n = 12; 9 multiparous and 3 primiparous), and retained placenta (n = 5 multiparous; Table 1). However, these health conditions were not acute and did not substantially decrease intake or cause continuous discomfort, including fever (>39.4°C) or labored breathing. Therefore, they were deemed to remain on trial. All cows received the same basal close-up diet once a day from −21 d to parturition (1.56 Mcal/kg DM and 14.46% CP). After calving, all cows received the same basal lactation diet once a day (1.76 Mcal/kg DM and 15.69% CP) until 100 DIM (Table 2). At −21 d before expected calving date, cows were assigned to either a basal control diet supplemented with 150 g/d ground corn (**CON**; n = 29) or the control diet supplemented with ground corn plus a rumen-derived DFM product (**GF**; n = 27; 150 g/d ground corn + 5 g/d of Galaxis Frontier [Native Microbials, San Diego, CA]; *Clostridium beijerinckii* at 1.0×10^7 cfu; *Pichia kudriavzevii* at 1.0×10^8 cfu; *Ruminococcus bovis* at 1.0×10^8 cfu; *Butyrivibrio fibrisolvens* at 1.0×10^8 cfu). These microorganisms were originally isolated from rumen content of high-yield dairy cows across a variety of feed regimens (Zengler and Embree, 2016) and prepared through a specialized encapsulation process to maintain live cell viability. Individual microorganisms were combined according to specific formulations by the manufacturer. The microbial additives were shipped from Native Microbials to South Dakota State University in individual daily-use sealed packages that were maintained under refrigerated conditions (~4°C) at all times. Individual amounts of treatments were weighed daily, along with 150 g of ground corn. Diets were fed as TMR and treatments were top-dressed individually once per day immediately after the morning feeding, and they were mixed by hand with the top portion of the TMR from −20 ± 5 to 100 DIM.

We evaluated the power of the study to detect statistically significant results on milk production using variance data from a previous transition cow study at South Dakota State University (Carpinelli et al., 2021). We found that 60 cows (30/treatment) would provide 80% power to detect a 3.7 kg/d difference in milk production.

Animal Management

Cows were enrolled in the experiment from March 2021 to February 2022. Before calving, cows were fed once a day at 0730 h using the individually feeding gate system (American Calan Inc., Northwood, NH). During the dry period, cows were housed in bedded-pack pens until parturition, at which point they were relocated into individual pens until 3 DIM. After 3 DIM, cows were moved to a freestall barn bedded with straw and assigned to an individual feeding gate (American Calan Inc., Northwood, NH). Lactating cows received the same basal lactation diet once a day at 0630 h until 100 d postpartum. Individual orts were collected once a day before morning feeding to determine intake. Feed offered was adjusted daily to achieve 5% to 10% refusals.

Diets were formulated using the Cornell Net Carbohydrate and Protein System model contained within the Agricultural Modeling and Training Systems (**AMTS**) CattlePro diet-balancing software (version 4.16.1, AMTS LLC, Groton, NY) to meet the requirements of the average cow in the group. After completion of the trial, diet formulation was evaluated with the NASEM (2021) model using the chemical analysis of feed ingredients (Table 2).

Body weight was measured weekly for each cow at 1200 h. Body condition score was recorded weekly by 2 trained evaluators using a 1 to 5 scale (1 = thin to 5 = obese) with increments of 0.25 units (Ferguson et al., 1994), and the average score was used for statistical analysis (Table 3).

Feed and Milk Samples

Individual samples of ingredients and TMR were collected once a week and stored at −20°C after DM analysis until further nutrient profile analysis. Samples

Table 2. Ingredient composition and chemical analysis of diets during the close-up (−21 d to calving) and lactation periods

Component	Diet	
	Close-up	Lactation
Ingredient,[1] % DM		
Corn silage	40.22	34.55
Alfalfa hay	—	14.58
Cottonseed	—	7.20
Molasses[2]	—	4.68
Grass hay	21.69	—
Wheat straw	10.14	2.70
Soybean meal	12.49	7.22
Distillers grains dry	3.67	1.58
Soybean hulls	3.22	0.23
Limestone	2.34	0.97
Biochlor[3]	1.89	—
Corn grain ground fine	1.33	18.07
Sodium bicarbonate	—	1.08
Magnesium sulfate	0.59	—
Rumen-inert fat[4]	—	0.70
Soy best[5]	—	5.08
Rumen-protected choline[6]	0.45	—
Calcium chloride	0.38	—
Magnesium oxide	0.38	0.22
Calcium sulfate	0.24	—
Urea	—	0.32
Biotin 2%	—	0.01
Calcium phosphate	0.17	0.22
Chromium 4%	0.14	—
Salt white	0.10	0.38
Vitamin E	0.24	0.04
Dairy vitamin premix[7]	0.17	0.09
Dairy TM premix[8]	0.10	0.09
Chemical analysis		
DM, %	49.8	50.6
NE_L, Mcal/kg DM	1.61	1.75
CP, % DM	14.6	15.8
NDF, % DM	45.0	31.2
ADF, % DM	29.2	20.5
Forage NDF, %	39.2	23.6
Starch, %	32.7	36.8
DCAD, mEq/100 g DM	−84	—

[1]Ingredients included in the ration formulated using AMTS.

[2]Quality liquid feeds (Dodgeville, WI; 60% DM and 38% sugar.

[3]Biochlor (Church & Dwight Co. Inc., Princeton, NJ).

[4]Energy Booster 100 (MSC, Carpentersville, IL).

[5]Grain State Soya, Inc., West Point, NE.

[6]Reashure (Balchem Corporation, New Hampton, NY).

[7]Contained 25.8% Ca (DM basis), 1,545 IU/kg of vitamin A, 387 IU/kg of vitamin D, and 4,826 IU/kg of vitamin E.

[8]TM = trace minerals; contained 11.7% Ca (DM basis), 1.96% S, 10,527 mg/kg of Fe, 63,158 mg/kg of Zn, 12,632 mg/kg of Cu, 63,158 mg/kg of Mn, 325 mg/kg of Se, 632 mg/kg of Co, and 1,053 mg/kg of I.

were dried in an oven at 105°C for 24 h, moisture loss was recorded, and this information was used to calculate DM content of the diet offered and refusals to calculate DMI. Composited monthly samples for ingredients were analyzed for DM (AOAC International, 2016; method 930.15), CP (total N × 6.25; AOAC International, 2016; method 990.03), NDF and ADF (Van Soest et al., 1991), and starch (YSI 2700 Select Biochemistry Analyzer, application note no. 319; YSI Life Sciences Inc.) by wet chemistry methods at a commercial laboratory (Dairy One, Ithaca, NY).

Cows were milked twice daily at 0530 h and 1630 h, and milk yield was recorded daily. Consecutive morning and evening milk samples were collected 1 d/wk during the experimental period. Milk samples were preserved (Broad Spectrum Microtabs II, Advanced Instruments) and morning and evening samples were analyzed separately for fat, protein, SCC, MUN, and lactose using Fourier-transform infrared spectroscopy technology (Dairy One). The ECM was calculated based on milk yield and milk sample analysis as follows: ECM = (12.51 × kg of fat/d) + (7.88 × kg of protein/d) + (5.32 × kg of lactose/d) (Hall, 2023).

Energy balance was calculated on an NE_L basis using equation 20-327 in NASEM (2021) as the supply minus all use. The NE_L intake (**NE_I**) was determined using daily DMI multiplied by NE_L density of the diet. The NE_L for maintenance (**NE_M**) was calculated as $BW^{0.75} \times 0.10$ (NASEM, 2021). The NE_L requirements for milk synthesis (**NE_{Milk}**) were calculated according to yields of fat, protein, and lactose based on NASEM (2021), as follows: $NE_{Milk} = (9.29 \times \text{fat yield}) + [5.85 \times \text{true protein yield} + (3.93 \times \text{lactose yield})]$. The NE_L for gestation (**NE_P**) was calculated using equation 3-18 in NASEM (2021) as $NE_P = (\text{GrUter\_Wt}_{gain}) \times 4.16$, where GrUter_Wt_{gain} is the gravid uterine wet tissue deposition gain estimated with equations 3-15a and 3-17a in NASEM (2021) as $\text{GrUter\_Wt}_{gain} = [0.0243 - (0.0000245 \times \text{Day of gestation})] \times (\text{calf birth weight} \times 1.825)$. The equations used to calculate prepartal energy balance (**EB_{PRE}**; Mcal/d) were $EB_{PRE} = NE_I - (NE_M + NE_P)$, and EB_{PRE} (as % of requirements) = $[NE_I/(NE_M + NE_P)] \times 100$. The equations used to calculate postpartal energy balance (**EB_{POST}**) were EB_{POST} (Mcal/d) = $NE_I - (NE_M + NE_{Milk})$, and EB_{POST} (as % of requirements) = $[NEI/(NE_M + NE_{Milk})] \times 100$.

For primiparous cows, the NE_M, NE_P, and NE_{Milk} were calculated with the same equations used for multiparous cows. Additionally, NE_L for frame growth (**NE_G**) was calculated using the equations 3-20c and 3-20e in NASEM (2021) as $NE_G = 9.4 \times \text{Fat\_ADG} + 5.55 \text{ Protein\_ADG}$. Equations used to estimate fat and protein in frame ADG were as follows: $\text{Fat\_ADG} = [0.067 + 0.375 \times (\text{BW/Mature BW})] \times \text{EBG/ADG}$, where EBG is the empty body weight gain, and $\text{Protein\_ADG} = [0.201 - 0.081 \times (\text{BW/Mature BW})] \times \text{EBG/ADG}$ (NASEM, 2021). Data from South Research and Training Facility (Brookings, SD) were used to obtain an estimate of mature BW of multiparous cows in the herd. Then, prepartal and postpartal energy balance (**EB**) for primiparous cows were calculated as $EB_{PRE} = NE_I - (NE_M + NE_P + NE_G)$ and $EB_{POST} = NE_I - (NE_M + NE_L + NE_G)$, respectively.

Table 3. Body weight, BCS, DMI, and energy balance responses in dairy cows fed a control diet (CON) or control diet plus direct-fed microbial (GF) during the peripartal period until 100 DIM

	Treatment			*P*-value			
Parameter	CON	GF	SEM[1]	Trt	Parity	Time	Trt × T[2]
Prepartum[3]							
BW, kg	713	715	16.4	0.88	<0.01	<0.01	0.51
BCS	3.41	3.47	0.03	0.14	—	0.03	0.58
DMI, kg/d	13.6	12.7	0.55	0.19	—	0.12	0.30
Energy balance, Mcal/d	0.62	−0.19	0.86	0.49	—	0.10	0.79
Energy balance, %	103.5	99.9	4.05	0.52	—	0.07	0.80
Postpartum[4]							
BW, kg	637	633	12.9	0.75	<0.01	<0.01	0.56
BCS	2.96	2.92	0.05	0.29	—	<0.01	0.93
DMI, kg/d	20.3	20.8	0.45	0.43	<0.01	<0.01	0.25
Energy balance, Mcal/d	−4.29	−4.50	0.61	0.79	<0.01	<0.01	0.95
Energy balance, %	89.3	89.0	1.48	0.88	<0.01	<0.01	0.89

[1]Largest SEM.

[2]Trt × T = interaction of treatment × time (week). None of the parameters had a parity × treatment effect ($P \geq 0.20$).

[3]Prepartum parameters, including weekly BW and BCS and daily DMI were analyzed from −21 d to calving.

[4]Postpartum parameters, including weekly BW and BCS and daily DMI were analyzed from calving to 100 DIM.

Blood Collection and Analyses

Blood was sampled from a coccygeal vessel before morning feeding using a 20-gauge needle (Beckton Dickinson, Franklin Lakes, NJ) at −22, −14, −7, 7, 14, and 30 d ± 2 relative to parturition from a random subset of multiparous cows (n = 12/treatment). Blood was collected into evacuated tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing either serum clot activator or lithium heparin. After blood collection, lithium heparin tubes were placed on ice, and tubes with serum clot activator were kept at 21°C until centrifugation (~30 min). Serum and plasma were obtained by centrifugation at 1,300 × *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 were analyzed for biomarkers of energy metabolism (i.e., glucose, nonesterified fatty acids [**NEFA**], and BHB), protein/N metabolism (i.e., urea, protein, and creatine), liver function (i.e., total bilirubin, glutamate oxaloacetate transaminase [**GOT**], gamma-glutamyl transferase [**GGT**], cholesterol, paraoxonase [**PON**], and albumin), inflammation (i.e., ceruloplasmin, haptoglobin [**HP**], and globulin), and oxidative stress (i.e., reactive oxygen metabolites [**ROM**] and ferric reducing antioxidant power) using kits purchased from Werfen Instrumentation Laboratory (Lexington, MA), and the procedures were described by Trevisi et al. (2012); Batistel et al. (2016); Jacometo et al. (2016).

Rumen Fluid Collection

Rumen fluid (~50 mL) was collected 3 to 4 h after feeding at −22, −14, −7, 1, 7, 14, 21, 70, and 90 d ± 2 relative to parturition from a random subset of multiparous cows (n = 12/ treatment) via esophageal tubing as described by Carpinelli et al. (2021). The first 200 mL were discarded to minimize saliva contamination, and approximately ~50 mL were collected. After collection, the ruminal pH was measured immediately using a pH meter (Oakton Instruments, Vernon Hills, IL). Two aliquots of 10 mL were transferred to bottles containing either 200 µL of 50% sulfuric acid or 2 mL of 25% metaphosphoric acid and stored at −20°C until analysis of ammonia N and VFA, respectively. Additionally, 2 mL of rumen fluid was immediately placed on ice and then transferred (~30 min) to liquid nitrogen and stored until DNA isolation. These DNA isolates were used for analysis of relative abundance of bacteria species via real-time quantitative PCR (**qPCR**).

Ammonia and VFA

Rumen fluid samples preserved with sulfuric acid were thawed and transferred into a 2-mL microcentrifuge tube and centrifuged at 30,000 × *g* for 20 min at 4°C (Model 5403, Eppendorf, Hamburg, Germany). The supernatant of the rumen fluid with sulfuric acid was used to analyze the ammonia N concentration using the assay described by Chaney and Marbach (1962). For the analysis of VFA concentrations, 0.11 mL of internal standard (5 mmol, 4-methyl-valeric acid, Sigma, St. Louis, MO) were added to thawed rumen fluid samples preserved with metaphosphoric acid (1 mL), vortexed, and rested for 30 min (4°C). Then, samples were centrifuged at 3,000 × *g* for 15 min at 4°C. 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). Next, 1 µL of the sample was injected at split 1:30, at injector temperature of 230°C. Separation of VFA profile was determined using Quadrex 007-10 Series (Quadrex Corp., New Haven, CT) 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 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.

Ruminal Bacteria DNA Isolation and qPCR Amplification of 16S rDNA Genes

Ruminal bacteria DNA was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with some modifications to the protocol outlined by Henderson et al. (2013). Briefly, 1 mL of rumen fluid was centrifuged at 12,000 × *g* for 5 min at 25°C. After, the supernatant was discarded, and the pellet was suspended in 1 mL of buffer EX, vortexed, and incubated in a heat block at 95°C for 5 min. Then, samples were centrifuged at 20,000 × *g* for 1 min at 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 15 s and incubated at 70°C for 10 min. After incubation, 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 have been previously validated and reported (Supplemental Table S2, see Notes). The relative abundance of 18 bacterial species was determined using qPCR analysis. The qPCR analysis was performed using 10 mL of qPCR mixture containing 4 mL of sample DNA, 5 mL of 1 × SYBR Green master mix (Applied Biosystems, Waltham, MA), 0.4 µL of 10 µ*M* 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 qPCR reactions were performed in a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using the same conditions described by Grazziotin et al. (2020). A geometrical mean of 2 universal bacteria primers was used to calculate the relative abundance of bacterial species (Abdelmegeid et al., 2018).

Statistical Analysis

Milk yield and feed efficiency (milk/DMI) data were analyzed throughout the study, and separately, periods of incremental EB (1–5 wk postpartum) and EB reaching a plateau between 90% and 100% (6–14 wk postpartum; Supplemental Figure S1, see Notes). Milk, DMI, and feed efficiency data were summarized by week for statistical analysis. Data were analyzed as repeated measures with the MIXED procedure of SAS (v. 9.4; 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} is the dependent, continuous variable; μ is the overall mean; D_i is the fixed effect of the ith treatment (i = 1 and 2); P_j is the fixed effect of the jth parity (j = 1, 2, 3); DP_{ij} is the fixed effect of ith treatment by the jth parity of the experiment interaction; 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 (day or week) 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. Interactions with parity were tested and removed from the model when $P > 0.30$. Repeated measure data with equally spaced data (e.g., milk yield and BW) were modeled by selecting the variance-covariance structures with the least Bayesian information criteria value among compound symmetry, autoregressive, or heterogeneous autoregressive. Blood biomarkers and rumen fluid data, including pH, VFA, NH_3, and relative abundance of microbial species, were analyzed at various time points that were not equally spaced; therefore, a spatial power covariance structure 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. Blood biomarkers and rumen fluid data on −22 d 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 $P < 0.20$. Health data were analyzed using the SAS FREQ procedure and interpreted using Fisher's exact

test probabilities. However, none of the health issues observed in this experiment were affected ($P = 0.14$) by treatment effects (Table 1). Outliers were excluded when the studentized residual exceeded an absolute value of 4. Statistical significance was declared at $P \leq 0.05$, and trends at $0.05 < P < 0.10$.

RESULTS

DMI, BW, BCS, and EB

Neither prepartal nor postpartal BW, BCS, DMI, and EB (Mcal/d or %) were affected ($P \geq 0.14$) by dietary treatments (Table 3).

Production Variables and Feed Efficiency

Although a trend ($P = 0.06$) for greater milk yield in GF cows compared with CON was observed during the entire experiment (Figure 1A), greater ($P = 0.02$; Figure 1E) milk yield was observed during the postfresh period in GF cows compared with CON. Feed efficiency, in terms of milk/DMI, tended ($P = 0.10$; Figure 1B) to be greater in GF cows compared with CON. Although feed efficiency was not affected ($P = 0.11$; Figure 1D) by GF supplementation during the fresh period, a greater ($P = 0.03$, Figure 1F) feed efficiency was observed in GF compared with CON during the postfresh period. The parity × treatment effect ($P = 0.05$) observed in feed efficiency in the fresh period was mainly associated with greater ($P = 0.04$; Supplemental Figure S2, see Notes) feed efficiency in GF compared with CON in primiparous cows. Milk composition parameters, including fat and protein, as well as MUN and SCC, were not affected by treatments ($P \geq 0.21$; Table 4).

Ruminal Fermentation

We observed a trend for treatment × time interaction (**Trt × T**; $P \leq 0.10$) for increased mole percentage of butyrate and caproate (Table 5). The Trt × T in butyrate was attributed to a greater ($P \leq 0.03$) butyrate mole percentage in GF cows compared with CON at 1 and 70 DIM (Supplemental Figure S3A, see Notes). The latter was reflected in an overall greater ($P = 0.04$) butyrate mole percentage in GF cows compared with CON across the trial period. The trend ($P = 0.06$) for the 3-way interaction parity × treatment × time observed in butyrate could be attributed to treatment differences occurring in second-lactation cows compared with third-lactation cows. In second-lactation cows, butyrate was greater ($P \leq 0.05$) in GF cows compared with CON at 1 and 70 DIM, and butyrate was lower in GF cows compared with CON at −7 DIM (Supplemental Figure S4A, see Notes). In the case of >third-lactation cows, butyrate was lower ($P = 0.02$) in GF cows compared with CON at 7 DIM (Figure S4B). The Trt × T in caproate was attributed to a greater ($P = 0.01$; Figure S3B) caproate mole percentage in GF cows compared with CON at 7 DIM. Additionally, we observed a trend for greater ($P \leq 0.10$) caproate in GF cows compared with CON at 1 and 14 DIM. A trend ($P \leq 0.10$) was observed for lower acetate and greater valerate in GF cows compared with CON. Ruminal pH, NH_3, total VFA, propionate, isobutyrate, isovalerate, and acetate/propionate ratio were not affected ($P \geq 0.43$) by treatment effects.

Abundance of Ruminal Bacteria

The *Anaerovibrio lipolytica* was the only bacteria with a Trt × T trend ($P = 0.08$; Table 6). This Trt × T was associated with a lower abundance of *A. lipolytica* in GF compared with CON at 7 ($P = 0.02$) and 100 DIM ($P < 0.01$; data not shown). Additionally, a trend for a greater abundance of *Megasphaera elsdenii* ($P = 0.07$) and *Prevotella albensis* ($P = 0.09$) was observed in GF cows compared with CON. In contrast to *M. elsdenii* and *P. albensis*, a trend for a lower ($P = 0.07$) abundance of *Fibrobacter succinogenes* was observed in GF cows compared with CON.

Blood Biomarkers

None of the biomarkers evaluated were affected by a Trt × T (Table 7). Glucose was lower ($P = 0.03$) in GF compared with CON. Additionally, a parity × Trt interaction ($P < 0.01$) observed in glucose was reflected in lower ($P = 0.01$) glucose levels in GF compared with CON in second-lactation cows but not in third-lactation cows (Supplemental Figure S5, see Notes). Greater GOT, ceruloplasmin, haptoglobin, and ROM were observed in GF cows compared with CON. Similar to glucose, paraoxonase tended ($P = 0.08$) to be lower in GF cows compared with CON.

DISCUSSION

Effects on DMI, BW, BCS, and EB

In the current study, GF supplementation did not affect prepartal and postpartal DMI, nor did it affect BW, BCS, or EB. Similarly, Dickerson et al. (2022) and Nehme Marinho et al. (2024) used the same product and dose in mid-lactation and early lactation cows, respectively, and did not observe any effects on DMI between DFM-supplemented cows and the control group. In contrast, Valldecabres et al. (2022) observed a greater DMI in DFM-supplemented cows at 0.33 g/kg

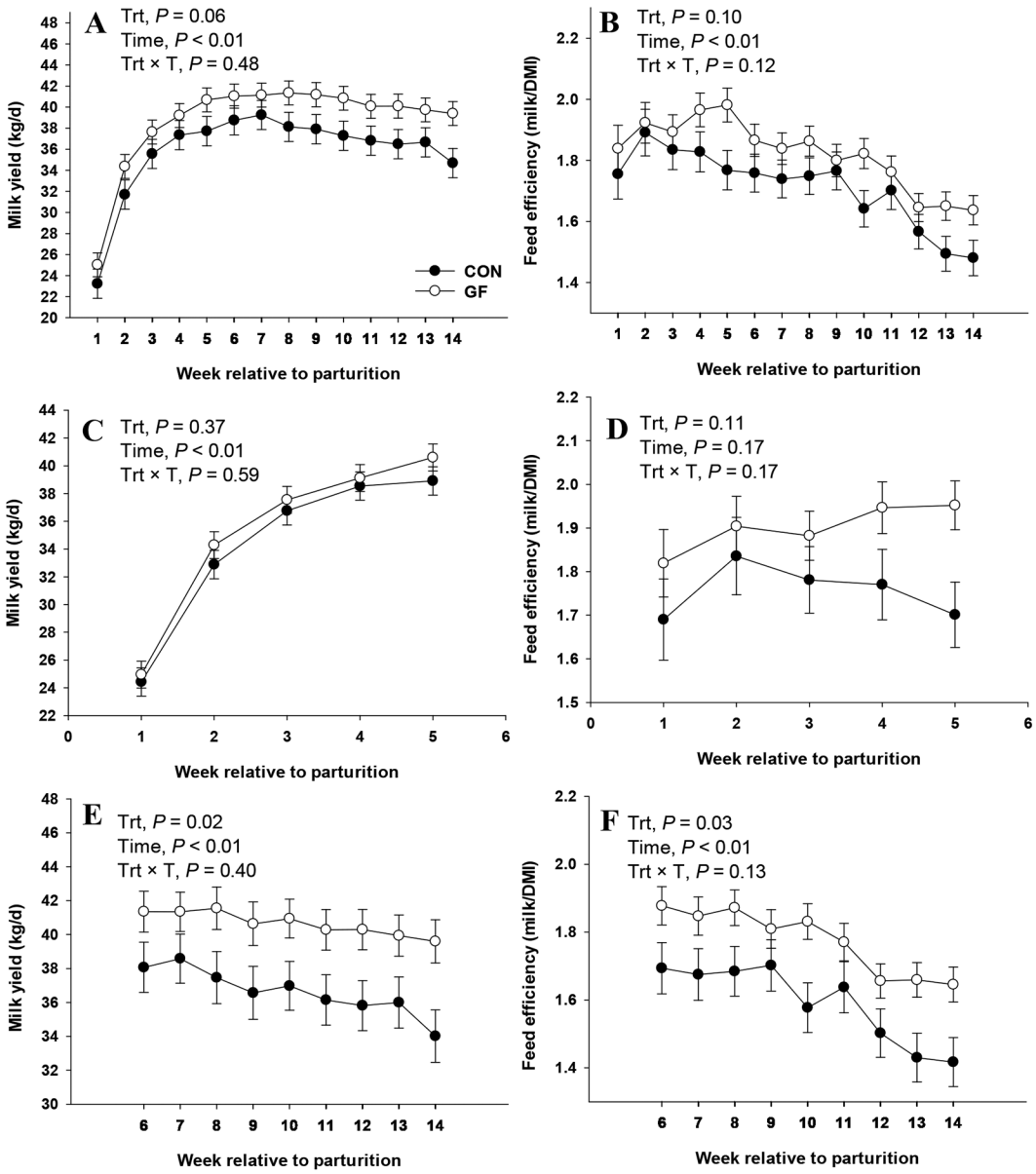

Figure 1. Milk yield (A), milk yield/DMI (B), early lactation milk yield (C), early lactation milk yield/DMI (D), mid-lactation milk yield (E), and mid-lactation milk yield/DMI (F) in dairy cows fed a control diet (CON) or control diet plus rumen-derived DFM (GF) from −21 ± 5 d relative to calving through 100 DIM. Trt × T = interaction of treatment × time. Values are means, with SE represented by vertical bars.

of TMR. This supplementation level was higher than the 0.24 g/kg of TMR dose used in the current study (assuming a 20.8 kg postpartal DMI in GF cows). Considering that GF cows in the current study were fed prepartum at a higher rate (0.39 g/kg of TMR) than those in Valldecabres et al. (2022), this does not seem to influence postpartal DMI. In contrast to our results, mid-lactation cows supplemented with a similar DFM and dose used in the current study had a trend (P = 0.08) for a decreased DMI (Goldsmith et al., 2023). Therefore, rumen-derived DFM seem to have limited to no effect on DMI.

Milk Production Parameters

The milk yield response in dairy cows to conventional and rumen-derived DFM has produced inconsistent results across studies. This is because commercially available DFM have multiple strains of fungi and bacteria (Ban and Guan, 2021), and many commonly used strains

are not naturally present in the rumen. In the case of rumen-derived DFM, previous studies indicate that the effect on milk production is related to the stage of lactation when cows are enrolled in the trial (Dickerson et al., 2022). Recent studies in early lactation reported that GF supplementation positively affects milk yield (1.5 and 3 kg/d; Valldecabres et al., 2022; Nehme Marinho et al., 2024). In contrast, other studies have reported a lack of effect on milk yield in mid-lactation cows supplemented with GF (Dickerson et al., 2022; Goldsmith et al., 2023). In the current study, a positive effect was observed in milk yield primarily during the postfresh period for GF cows, producing 4.1 kg/d more milk than CON. This was similar to improvements in milk production (3 kg/d) observed in cows after 16 wk of GF supplementation (Valldecabres et al., 2022). The authors associate these effects with a required time for GF strains to integrate with the pre-existing ruminal microbial population. Similar to results reported by Valldecabres et al. (2022), we observed improvements in milk yield during mid-lactation after GF cows had been supplemented for 3 wk prepartum and 5 wk postpartum. This delayed response seems to underscore the need for an integration time, as suggested by Valldecabres et al. (2022), allowing the GF microorganisms to drive a new microbiome dynamic that can impact metabolic function and consequently improve milk production.

Although Valldecabres et al. (2022) reported greater milk fat and protein yield in GF-supplemented cows compared with control, no differences were observed in fat and protein percentage. In contrast, other studies on GF supplementation have reported no effects on milk fat and protein percentage or yield in mid-lactation cows (Goetz et al., 2021; Dickerson et al., 2022; Goldsmith et al., 2023). The latter is consistent with our results, where no significant effects were observed in milk fat and protein percentage or yield.

Improvements in feed efficiency have been previously reported when supplementing early lactation (Valldecabres et al., 2022) and mid-lactation cows with rumen-derived DFM (Dickerson et al., 2022; Goldsmith et al., 2023). Our results are consistent with these findings, where we observed an increase in feed efficiency in terms of milk/DMI in GF cows compared with CON during postfresh period. The latter can be mainly attributed to an increase in milk production during mid-lactation (Table 4) because postpartal DMI was not affected by GF supplementation (Table 3).

Rumen Fermentation Parameters

The rumen microbiome is responsible for feed digestion, fermenting dietary carbohydrates into metabolic end products, including VFA (e.g., acetate, propionate, and butyrate; Shabat et al., 2016; Clemmons et al., 2019). Inconsistent results on total VFA have been reported when supplementing non-rumen-derived DFM to dairy cows. A meta-analysis conducted by Desnoyers et al. (2009) found that supplementing yeast led to a significant increase in total VFA. Similarly, a recent study reported positive effects on total VFA when dairy cows were supplemented with a *Saccharomyces cerevisiae*–based DFM (Oh et al., 2019). In contrast, others have observed lower or no response on total VFA when supplementing *S. cerevisiae*–based products as DFM to lactating dairy cows (Thrune et al., 2009; Bayat et al., 2015; Philippeau et al., 2017). Similarly, in the current study, rumen-derived DFM supplementation did not affect total VFA.

Current studies exploring the impact of a DFM composed of *C. beijerinckii*, *P. kudriavzevii*, *R. bovis*, and *B. fibrisolvens* have not reported effects on ruminal fermentation end products in dairy cows (Goetz et al., 2021; Dickerson et al., 2022; Goldsmith et al., 2023). However, individual assessment of these microorganisms revealed that *C. beijerinckii* and *B. fibrisolvens* primarily produce butyrate, whereas *R. bovis* predominantly generates acetate (Emerson and Weimer, 2017; Gomez-Flores et al., 2017; Gaffney et al., 2021). These findings are in line with our results suggesting that, to some extent, supplementing dairy cows with GF increases butyrate levels (Table 5). However, this effect seems to be minimal and derived from sporadic increments in butyrate in GF cows compared with CON at 1 and 70 DIM (Supplemental Figure S3A). In contrast to butyrate, acetate tended to be lower in GF cows, suggesting a limited or no influence of acetate-producers *R. bovis* and *P. kudriavzevii* in the GF supplement on ruminal fermentation (Gaffney et al., 2021; Suntara et al., 2021).

Ruminal Bacterial Species

Dairy cows are expected to experience a shift in ruminal microbial populations around parturition. This can be attributed to the natural adaptation of ruminal microbes to a lactating cow diet after calving. During the transition period, dairy cows are commonly switched from a predominantly forage-based diet to a high-grain diet (Clemmons et al., 2019). Minuti et al. (2015) suggested that diet alterations after calving could reduce the number of fiber-digesting bacteria.

Bacterial DFM have been shown to increase ruminal lactic acid–utilizing bacteria (**LUB**) and decrease lactic acid-producing bacteria, potentially leading to a reduction in ruminal acidosis. On the other hand, fungal DFM have been reported to stimulate the growth of various ruminal bacterial populations, including fibrolytic bacteria, LUB, and amylolytic bacteria (Ban

Table 4. Milk production and composition responses in dairy cows fed a control diet (CON) or control diet plus direct-fed microbial (GF) during the peripartal period until 100 DIM

	Treatment			*P*-value			
Item	CON	GF	SEM[1]	Trt	Parity[2]	Time	Trt × T[3]
Milk yield, kg/d	35.8	38.7	1.23	0.06	0.03	<0.01	0.48
Fresh period	34.3	35.3	0.90	0.37	<0.01	<0.01	0.59
Postfresh period	36.6	40.7	1.39	0.02	0.05	<0.01	0.40
Milk composition							
Fat %	3.37	3.36	0.06	0.94	—	<0.01	0.17
Protein %	3.14	3.07	0.04	0.21	—	<0.01	0.28
SCC[4]	1.52	1.72	0.29	0.47	0.14	<0.01	0.89
MUN	9.42	9.71	0.37	0.43	—	<0.01	0.27
Yield of milk components							
Milk fat yield, kg/d	1.25	1.30	0.03	0.33	—	<0.01	0.39
Milk protein yield, kg/d	1.14	1.18	0.03	0.24	<0.01	<0.01	0.31
ECM, kg/d	33.7	34.8	0.80	0.23	<0.01	0.03	0.36
Milk/DMI, kg/kg	1.71	1.82	0.05	0.10	—	<0.01	0.12
Fresh period	1.84	1.94	0.05	0.11	—	0.17	0.17
Postfresh period	1.59	1.77	0.07	0.03	0.17	<0.01	0.13

[1]Largest SEM.

[2]A parity × treatment effect ($P = 0.05$) was observed in milk efficiency (milk/DMI) in the fresh period, all other parameters did not have a parity × treatment effect ($P \geq 0.23$).

[3]Trt × T = interaction of treatment × time (week).

[4]SCC were transformed to Log10.

and Guan, 2021). Currently, there is a lack of literature examining the potential influence of the yeast (*P. kudriavzevii)* and bacterial species (*B. fibrisolvens*, *C. beijerinckii*, and *R. bovis)* used in GF on ruminal microbial abundance in transition dairy cows.

A study reported that supplementation of non-rumen-derived live yeast in early lactation stimulated the lactate-utilizing bacteria *M. elsdenii*, which helped counteract the effects of SARA (Pinloche et al., 2013). In the present study, lactate levels were not measured; however, a trend was observed for a greater abundance of *M. elsdenii.* This species is a known lactate utilizer for producing propionate and butyrate and was reported to be highly enriched in the rumen of highly efficient dairy cows (Shabat et al., 2016). The observed trend for increased *M. elsdenii* in GF cows may have partially contributed to the trends reported for improved efficiency in GF cows.

Fibrobacter succinogenes has been recognized as a major ruminal cellulolytic bacteria involved in active hemicellulose hydrolysis and utilization (Emerson and Weimer, 2017; Jiang et al., 2017). The *S. cerevisiae* supplementation in cows fed low-quality forage or under SARA conditions has been reported to increase the abundance of *F. succinogenes* (AlZahal et al., 2014; Malekkhahi et al., 2016; Amin and Mao, 2021). In contrast, other studies reported no effect of non-rumen-derived live yeast supplementation on ruminal bacterial abundance of *F. succinogenes* (Silberberg et al., 2013; Bayat et al., 2015). Minuti et al. (2015) reported that the abundance of *F. succinogenes* decreased in transition dairy cows after calving, mainly attributed to the high grain content in the lactation diet. A trend ($P = 0.07$) for lower *F. succinogenes* in GF compared with control was observed in this study, suggesting that the DFM failed to stimulate or maintain the growth of this fiber digester. The observed trend for lower abundance of *F. succinogenes* may have partially contributed to the trend for lower acetate mole percentage in GF cows.

Studies have reported that at the genus level, *Prevotella* are the most abundant bacteria in the rumen, accounting for as much as 60% of the bacterial community (Bekele et al., 2010; Clemmons et al., 2019; Carpinelli et al., 2021). However, the majority of *Prevotella* spp. in rumen remain uncultured because only 2% to 4% of the bacterial rRNA gene copies were represented by the known *Prevotella* species (*Prevotella bryantii*, *Prevotella ruminicola*, and *Prevotella brevis*; Stevenson and Weimer, 2007). *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). In the current study, response due to GF supplementation was only observed for *P. albensis*, with a trend for increased abundance in GF cows.

Taken together, our data suggest that GF supplementation promoted mild changes in bacterial abundance of amylolytic, cellulolytic, and lactate utilizers in the rumen. These changes have limited effects on ruminal fermentation. For instance, the significant change in butyrate can be considered biologically negligible. However, these small changes could be potentially related to large diurnal differences between ruminal fermentation in GF cows and control. This can be attributed to the

Table 5. Rumen fermentation characteristics responses in dairy cows fed a control diet (CON) or control diet plus direct-fed microbial (GF) during the peripartal period until 100 DIM

	Treatment			*P*-value		
Parameter	CON	GF	SEM[1]	Trt	Time	Trt × T[2]
pH	6.5	6.5	0.05	0.43	<0.01	0.82
NH_3, mg/dL	10.4	10.2	0.57	0.79	<0.01	0.69
Total VFA, n*M*	113.8	113.0	3.32	0.87	0.04	0.65
VFA, mol/100 mol						
Acetate, %	72.3	71.2	0.55	0.10	<0.01	0.77
Propionate, %	17.0	17.4	0.54	0.47	<0.01	0.69
Butyrate, %	7.4	7.9	0.19	0.04	0.01	0.10
Valerate, %	1.2	1.3	0.04	0.06	0.05	0.64
Caproate, %	0.6	0.6	0.02	0.52	<0.01	0.09
Iso-butyrate, %	0.9	0.9	0.02	0.71	<0.01	0.81
Iso-valerate, %	0.7	0.7	0.02	0.73	<0.01	0.48
Acetate:propionate	4.5	4.4	0.17	0.60	<0.01	0.59

[1]Largest SEM.

[2]Trt × T = interaction of treatment × time (d). None of the parameters had a parity ($P \geq 0.31$) or a parity × treatment effect ($P \geq 0.66$). A trend ($P = 0.06$) for a parity × treatment × time was observed for butyrate.

fact that in the present study, rumen fluid collection was taken between 3 and 4 h after feeding, which represents a single snapshot of a 24-h dynamic rhythm of microbial growth and synthesis and absorption of fermentation end products.

Blood Biomarkers of Energy Metabolism

During the transition period, the contrast between insufficient energy intake and high energy demand for milk synthesis results in NEB (Ingvartsen and Andersen, 2000). During early lactation, plasma glucose concentrations are reduced (Ingvartsen and Andersen, 2000). The latter is partially explained by a high demand for glucose for lactose synthesis, an essential milk component commonly regarded as the limiting factor for milk synthesis. Additionally, there may be insufficient rates of hepatic gluconeogenesis to meet the high glucose demand (Zarrin et al., 2017).

Currently, there is a lack of research that examines the potential influence of rumen-derived DFM on homeorhetic adjustments during the peripartal period of dairy cows. However, research in mid-lactation cows reported no effect of GF supplementation on blood glucose (Goldsmith et al., 2023). Inconsistent responses on blood glucose have been reported when supplementing other bacterial or fungal DFM not evaluated in the current study (Oetzel et al., 2007; AlZahal et al., 2014). For instance, transition dairy cows supplemented with *S. cerevisiae* and 2 strains of *Enterococcus faecium* exhibited significantly elevated glucose and insulin concentrations (Oetzel et al., 2007). In the present study, GF-supplemented cows showed 3% lower blood glucose than control (4.28 mmol/L vs. 4.42 mmol/L), with nadir levels observed at 7 DIM for both groups (Supplemental Figure S6A, see Notes). A similar postpartal decline in glucose was reported by Mezzetti et al. (2019), followed by a steady increase through 28 DIM. In the current study, the same steady increase in glucose was observed, regardless of treatment, through 30 DIM, suggesting a similar metabolic adaptation to both groups. It is noteworthy that the postpartal decrease in glucose in this study remained within physiological levels observed in transition dairy cows (Wankhade et al., 2017; Dubuc and Buczinski, 2018; Mezzetti et al., 2019).

Blood Biomarkers of Liver Function and Inflammation

During the transition period, dairy cows normally experience an inflammatory condition characterized by the release of proinflammatory cytokines (Bertoni et al., 2008). These cytokines can prompt a systemic response by modulating the synthesis of specific proteins in the liver, called acute phase proteins (**APP**). These APP are further classified as positive APP (**+APP**) and negative APP (**−APP**; Bertoni et al., 2008). The +APP refers to liver proteins that are synthesized at a greater rate during a systemic response or acute phase response, including ceruloplasmin and HP. In contrast, −APP represents a rate synthesis decrease in liver proteins, such as albumin, retinol-binding proteins, and apolipoproteins (Bionaz et al., 2007; Bertoni et al., 2008).

The role of ceruloplasmin and HP as +APP include protection against pathogens by capturing minerals (e.g., Fe and Zn) in blood to reduce pathogen survival (Trevisi and Minuti, 2018). Moreover, among +APP, HP is one of the most commonly used biomarkers of inflammation in dairy cows due to its longer plasma half-life relative to other +APP (Mezzetti et al., 2020). The HP is an α-globulin constituent that binds to free hemoglobin, which is toxic

Table 6. Relative abundance (%) of target bacterial species mixed ruminal fluid from peripartal dairy cows fed a control diet (CON) or control diet plus direct-fed microbial (GF) during the peripartal period until 100 DIM

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

[1]Data were log-transformed before statistics. The SEM associated with log-transformed data are in log scale.

[2]Largest SEM is shown.

[2]Trt × T = interaction of treatment × time (day). None of the parameters had a parity × treatment effect ($P > 0.30$).

and proinflammatory; it is present in the plasma and reduces the oxidative damage associated with hemolysis (Murata et al., 2004). In cattle, HP has been reported to be effective in diagnosing and prognosis diseases (e.g., mastitis and endometritis; Murata et al., 2004).

Ceruloplasmin is a copper-containing protein that oxidizes toxic ferrous iron to its nontoxic ferric form (Patel et al., 2002). Ceruloplasmin is mainly synthesized in the liver. It protects tissues from iron-mediated free radical injury and is involved in various antioxidant and cytoprotective activities (Murata et al., 2004). Unlike HP, the application of ceruloplasmin in diagnosis is less common; however, evidence suggests that it can indicate an infection in cattle and other species (Murata et al., 2004). Often, ceruloplasmin is correlated with markers of oxidative stress, inflammation, and the innate immune system (Trevisi and Minuti, 2018). Previous studies have reported increased levels of HP and ceruloplasmin around parturition (Bertoni et al., 2008; Trevisi et al., 2012; Mezzetti et al., 2020).

In the current study, GF cows showed higher levels of HP and ceruloplasmin when compared with CON (0.21 g/L vs. 0.14 g/L and 3.21/L µmol/L vs. 2.95 µmol/L, respectively). In contrast, some studies supplementing DFM not part of the evaluated GF supplement (i.e., *S. cerevisiae* with *E. faecium*, *Bacillus pumilus*, or *S. cerevisiae* var. *boulardii*) during the transition period reported no treatment effect on HP (AlZahal et al., 2014; Luan et al., 2015; Hiltz et al., 2023). Similarly, a lack of effect on ceruloplasmin has been reported in transition cows supplemented with *S. cerevisiae* (Cattaneo et al., 2023). The increase of both +APP around parturition, regardless of treatment, reached zenith levels at 7 DIM (Supplemental Figure S6C–D) and is in agreement with a physiological inflammatory-like condition experienced by transition cows soon after calving (Bertoni et al., 2008; Trevisi et al., 2012). However, HP concentrations at 7 DIM were below under values reported for low liver function cows and ketotic cows (Bertoni et al., 2008; Mezzetti et al., 2019). This mild increase in HP and ceruloplasmin in GF may suggest a more active acute phase response in these cows. This effect, coupled with the similar milk yield and DMI in GF cows compared with control, indicates that this increased inflammation was not detrimental to either lactation performance or health. Regardless of treatment, the rapid decline in ceruloplasmin and HP after 7 DIM suggests that cows in this current experiment had a normal resolution of inflammation postpartum.

Glutamate oxaloacetate transaminase is an enzyme involved in hepatic AA metabolism, and high concentrations of this biomarker in blood indicate liver damage. Mezzetti et al. (2019) observed that GOT concentration reached maximal levels at 7 DIM, around 130 U/L in ketotic cows, whereas GOT in healthy cows remained under 110 U/L. After 7 DIM, Mezzetti et al. (2019) observed a decrease in GOT in both groups until 28 DIM. Although GOT was greater in GF cows compared with CON, the levels of GOT at 7 DIM were above 110 U/L for both groups. After 7 DIM, we observed a similar reduction in GOT, as reported by Mezzetti et al. (2019). The lack of

Table 7. Blood biomarkers related to energy and nitrogen metabolism, liver function, inflammation, and oxidative stress responses in dairy cows fed a control diet (CON) or control diet plus a direct-fed microbial (GF) during the peripartal period until 100 DIM

	Treatment			*P*-value			
Parameter	CON	GF	SEM[1]	Trt	Parity[2]	Time	Trt × T[3]
Energy metabolism							
Glucose,[4] mmol/L	4.42	4.28	0.02	0.03	0.05	0.14	0.82
NEFA,[4] mmol/L	0.15	0.19	0.18	0.18	—	<0.01	0.82
BHB,[4] mmol/L	0.44	0.45	0.10	0.67	—	<0.01	0.65
Nitrogen metabolism							
Urea, mmol/L	4.56	4.57	0.19	0.96	0.23	<0.01	0.36
Protein, g/L	73.4	73.7	0.91	0.76	0.27	<0.01	0.74
Creatinine, µmol/L	89.0	88.4	1.48	0.68	—	<0.01	0.74
Liver function							
Total bilirubin, µmol/L	2.27	2.45	0.15	0.37	0.12	<0.01	0.99
GOT, U/L	94.8	103.2	2.69	0.02	—	<0.01	0.89
GGT, U/L	20.5	19.3	0.88	0.33	—	<0.01	0.89
Cholesterol, mmol/L	3.13	3.08	0.08	0.62	—	<0.01	0.66
Paraoxonase, U/mL	85.2	78.9	2.83	0.08	—	<0.01	0.65
Albumin, g/L	34.1	33.4	0.39	0.14	0.30	<0.01	0.35
Inflammation and acute phase proteins							
Ceruloplasmin, µmol/L	2.95	3.21	0.10	0.01	0.21	<0.01	0.68
Haptoglobin,[4] g/L	0.14	0.21	0.24	0.03	—	<0.01	0.75
Globulin, g/L	39.4	40.2	0.86	0.35	0.18	<0.01	0.81
Oxidative stress							
FRAP,[5] µmol/L	136.1	135.5	2.54	0.85	—	<0.01	0.13
ROM, mg of H_2O_2/100 mL	15.7	17.4	0.47	0.01	—	<0.01	0.99

[1]Largest standard error of the mean is shown.

[2]A parity × treatment effect ($P < 0.01$) was observed in glucose, and a trend ($P = 0.07$) for a parity × treatment was observed in paraoxonase.

[3]Trt × T = interaction of treatment × time (d).

[4]Data were log-transformed before statistics. The standard errors of the means associated with log-transformed data are in log scale.

[5]FRAP = ferric reducing antioxidant power.

differences in other biomarkers of liver function, such as albumin and GGT, does not provide a conclusive scenario of liver damage in GF cows; however, the positive effects of GF on milk production while maintaining similar DMI to control cows indicate that inflammatory-like conditions experienced by GF cows were not detrimental.

Biomarkers of Oxidative Stress

Oxidative stress is the result of an imbalance between ROM production and the neutralizing capacity of antioxidant mechanisms (Abuelo et al., 2015). Additionally, oxidative stress can modify important physiological and metabolic functions, leading to alterations in transition cow physiology and increasing risk for additional pathologies (Bernabucci et al., 2005). The production of ROM is part of a normal biological process such as β-oxidation and phagocytosis. For instance, phagocytic cells use ROM to kill bacteria (Abuelo et al., 2015). In the liver, ROM are produced during fatty acid β oxidation, which implies that elevated NEFA in early lactation leads to a corresponding increase in ROM (Mezzetti et al., 2020).

In the current study, GF cows showed greater ROM concentration when compared with CON cows. Regardless of treatment, ROM concentrations peaked at 7 DIM, which agrees with findings observed previously by Bionaz et al. (2007) and Mezzetti et al. (2019). Similar to ceruloplasmin and HP, ROM concentrations decreased rapidly after 7 DIM, which indicates a decline in oxidative stress exposure and, consequently, a lower risk of developing diseases postpartum. This is well demonstrated by Mezzetti et al. (2019), where ROM concentration remained >16 from 3 to 28 DIM in cows diagnosed with subclinical ketosis. This same group of ketotic cows had higher NEFA and BHB levels after calving. Although GF cows in the current study had greater ROM concentration throughout the transition period, ROM levels were under 16 by 30 DIM (Supplemental Figure S6E), suggesting a lower risk for oxidative stress, which is confirmed by the similar NEFA and BHB concentrations between GF and CON cows.

According to Turk et al. (2005), reduced levels of PON may result in a decline in antioxidative protection during early lactation. Additionally, PON is inversely cor-

related with oxidative stress, partly due to its ability to protect both low-density lipids and high-density lipids from lipid peroxidation. Bionaz et al. (2007) stratified transition dairy cows based on plasma PON concentrations into quartiles upper (92.0 ± 19.8), intermediate upper (67.8 ± 13.0), intermediate lower (54.3 ± 11.7), and lower groups (43.8 ± 12.7). They observed that the lower group maintained ROM levels >16 from calving until 28 DIM and reduced PON levels <70 U/mL until 63 DIM. In the current study, a contrasting effect of increased ROM with a trend for a decreased PON in GF cows indicates a plausible oxidative stress condition. However, as mentioned above, ROM was lower than 16 by 30 DIM in GF cows while maintaining a decent PON concentration >70 after 14 DIM (data not shown). The latter, coupled with the improved milk yield in GF cows while maintaining a similar DMI to CON cows, suggest that GF oxidative status and mild inflammatory condition were not detrimental. Abuelo et al. (2015) indicated that a certain level of inflammation after calving is not a pathological process but rather an adaptive process necessary for a successful transition into lactation. To our knowledge, there is a lack of data on DFM supplementation effects on oxidative stress. Hence, further research is needed to clearly elucidate the effects of DFM on the redox balance in transition dairy cows.

CONCLUSIONS

The findings of this study revealed that supplementation of a rumen-derived DFM composed of *C. beijerinckii*, *P. kudriavzevii*, *R. bovis*, and *B. fibrisolvens* (GF), promoted positive responses in lactation performance during the postfresh period, such as milk yield and feed efficiency in terms of milk/DMI. In the rumen, GF contributed to transient increments of butyrate after calving. The GF supplementation may have induced a more heightened metabolism postpartum that was accompanied by increased ROM and a mild inflammatory condition in GF cows. However, the improved milk yield in GF cows while maintaining a similar DMI to CON, suggest that GF oxidative status and mild inflammatory condition were not detrimental. The effects of DFM on health and performance in transition dairy cows and its mechanisms remain to be elucidated. The findings in this study indicate that supplementing GF during the transition period through the postfresh period positively affects lactation performance.

NOTES

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Nonstandard abbreviations used: +APP = positive APP; AMTS = Agricultural Modeling and Training Systems; APP = acute phase proteins; −APP = negative APP; CON = basal control diet; DFM = direct-fed microbial; EB = energy balance; EB_{POST} = postpartal EB; EB_{PRE} = prepartal EB; FRAP = ferric reducing antioxidant power; GF = control diet plus a rumen-derived DFM product; GGT = gamma-glutamyl transferase; GOT = glutamate oxaloacetate transaminase; HP = haptoglobin; LUB = lactic acid–utilizing bacteria; NEB = negative energy balance; NEFA = nonesterified fatty acids; NE_G = NE_L for frame growth; NE_I = NE_L intake; NE_M = NE_L for maintenance; NE_{Milk} = NE_L requirement for milk synthesis; NE_P = NE_L for gestation; PON = paraoxonase; qPCR = quantitative PCR; ROM = reactive oxygen metabolites; TM = trace minerals; Trt × T = treatment × time interaction.

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