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Effects of supplementing a direct-fed microbial containing *Enterococcus faecium* 669 on performance, health, and metabolic responses of preweaning Holstein dairy calves

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ABSTRACT

This study aimed to evaluate the effects of *Enterococcus faecium* 669 supplementation on performance, health, parasitological, microbiological, and hematological responses of preweaning dairy calves. Forty-two newborn Holstein female calves (initial body weight [BW] 44 ± 4.5 kg) were used in the present study. At birth, calves were ranked by initial BW and assigned to 1 of 2 treatment groups: (1) whole milk (CON; n = 21) and (2) whole milk with the addition of direct-fed microbial *Ent. faecium* 669 (DFM; n = 21). During the entire experimental period (63 d), DFM was daily-fed at a rate of 2.5×10^9 cfu/head. All calves were offered a mixture of a starter feed and wheat straw for ad libitum consumption. Supplement intake was evaluated daily, whereas calves were weighed on a weekly basis from d 0 to weaning (d 63). Diarrhea was assessed once a day, and fecal and blood samples were collected for microbiological, parasitological, and hematological responses. All data were analyzed with SAS using calf as the experimental unit. A treatment × week interaction was observed for BW, as DFM-supplemented calves were heavier than CON cohorts on d 56 (+ 4.7 kg) and at weaning on d 63 (+ 4.8 kg). A similar interaction was observed for average daily gain (ADG) and dry matter intake (DMI), with greater ADG for DFM-supplemented calves from d 35 to 42, greater ADG and DMI from d 49 to 56, and greater DMI from d 56 to weaning. Moreover, diarrhea occurrence tended to be lower, whereas rectal temperature was 0.2°C lower for DFM-supplemented calves. Treatment × day interactions were observed for the occurrence and counts of *Eimeria* spp., as DFM-supplemented calves tended to have a reduced number of positive observations on d 42 of the study versus CON, and a significant reduction in positive animals from d 21 to 42 was observed in the DFM group but not in CON calves. For *Cryptosporidium* spp., no treatment effects were observed on overall occurrence (%), but DFM-supplemented calves had a greater count of oocyst per gram versus CON. No treatment × day interaction or main treatment effects were observed for any of the blood variables analyzed herein, exception being monocytes concentration. In summary, preweaning *Ent. faecium* 669 supplementation improved performance, diarrhea occurrence, and reduced the number of calves positively-detected for *Eimeria* spp.

Key words: diarrhea, direct-fed microbial, *Eimeria* spp., *Enterococcus faecium* 669, preweaning dairy calves

INTRODUCTION

Dairy calf management may determine medium- and long-term productive performance of the dairy cattle herd. For example, Bach et al. (2021) reported a positive relationship between initial 70-d ADG of heifer calves and milk production in the first lactation when using data from a large Spanish dairy operation. However, adverse health events, such as diarrhea, often deplete preweaning performance, likely avoiding the achievement of the full productive potential of the herd, with negative effects that last until later in life (Soberon et al., 2012). In fact, diarrhea is one of the most prevalent adverse health events reported in different regions of the world, including United States, Canada, and Australia (NAHMS, 2007, 2014; Windeyer et al., 2014; Abuelo et al., 2019). Hence, technologies that support the health of preweaning dairy calves, while also improving their growth rates, are warranted and should be further evaluated.

One of these technologies that have been used and gaining interest in the dairy production settings is the direct-fed microbials (**DFM**), also known as probiotics. To be classified as a DFM, the live bacteria must support the health of the herd (FAO and WHO, 2001; Mar-

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kowiak and Śliżewska, 2018). Different bacteria have been used as DFM strains for ruminants, but focus has been mostly on *Lactobacillus* spp., *Bacillus* spp., and *Enterococcus* spp. More specifically on *Enterococcus* spp., no safety concerns regarding transmission of virulence genes and spread of these genes to humans have been unraveled (Shridhar et al., 2022), supporting the results from Cappellozza et al. (2023). These authors recently demonstrated that supplementing preweaning dairy calves with *Enterococcus faecium* 669 tended to reduce the occurrence of diarrhea, while supporting a greater growth in these animals. However, no other variables related to health, such as microbiological and parasitological counts, fecal pH, fecal fermentation profile, were evaluated by Cappellozza et al. (2023). Based on this rationale, we hypothesized that feeding *Ent. faecium* 669 would reduce the occurrence of diarrhea and improve performance of preweaning Holstein dairy calves. Hence, our objective was to evaluate the effects of *Ent. faecium* 669 supplementation on health, microbiological and parasitological counts, as well as performance of preweaning Holstein dairy calves.

MATERIALS AND METHODS

The present experiment was conducted at the Bursa Uludag University (Bursa, Türkiye) from February to June 2022. All animals used in both experiments were cared for in accordance with the practices outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Training (FASS, 2010) and the experimental activities approved by the Institutional Animal Care and Use Committee from the Bursa Uludag University (B.30.2.ULU.0.8Z.00.00/2022–01/05).

Animals, Management, Treatments, and Housing

Forty-two newborn Holstein female calves [initial BW 44 ± 4.5 kg] were used in the present study. At birth, calves were ranked by initial BW and assigned to 1 of 2 treatment groups: (1) daily administration of whole milk without DFM supplementation (**CON**; n = 21) and (2) daily administration of whole milk with the addition of *Ent. faecium* 669 (Lactiferm; Chr. Hansen A/S, Hørsholm, Denmark; **ENT**; n = 21). During the entire experimental period (63 d), DFM was mixed directly in the whole milk and daily-fed at a rate of 1 g/head (2.5×10^9 cfu/head per day). Moreover, all animals were individually fed 6 L of whole milk twice a day (3 L/feeding) from d 4 to 55, whereas the gradual weaning management started on d 56 and consisted of reducing the amount of whole milk offered by 2 L until d 63 (weaning). The whole milk offered herein was analyzed with the Milko-Scan FT1 analyzer (Foss Electric, Hillerød, Denmark) and contained 12.1% DM, 3.55% fat, 3.13% protein, and 5.4% lactose.

Table 1. Composition and nutritional profile of the mixture containing the starter supplement and wheat straw offered to all calves during the present experiment[1]

Item	Calf starter	Wheat straw
DM, %	89.4	94.3
CP, % DM	23.7	5.1
Ether extract, % DM	3.2	1.6
Ash, % DM	8.3	6.7
Starch, % DM	26.7	3.0
NDF, % DM	26.1	70.7
ADF, % DM	8.5	39.2
ADL, % DM	10.5	52.8

[1]Vitamin-mineral premix was added to the calf starter and contained, per kg of supplement, 200,000.00 IU of vitamin A, 30,000.00 IU of vitamin D, 25,000 mg of vitamin E, 4,000 mg of vitamin B_1, 8,000 mg of vitamin B_2, 5,000 mg of vitamin B_6, 20 mg of vitamin B_{12}, 20,000 mg of niacin, 200,000 mg of choline chloride, 50,000 mg of Mn, 50,000 mg of Zn, 20,000 g of Mg, 50,000 mg of Fe, 150 mg of Co, 10,000 mg of Cu, 800 mg of I, and 150 mg of Se.

Following colostrum administration at birth (quality ≥23 mg of IgG/dL), calves were taken to individual hutches (1.65 × 1.20 m), where calves were reared until weaning on d 63. All colostrum offered to the calves was evaluated as failure in transfer passive immunity has been associated with significant calf health and growth (Elsohaby et al., 2019; Crannell and Abuelo, 2023), as well as future reproductive performance (Crannell and Abuelo, 2023). The whole milk offered during the experimental period did not contain any antibiotic residue, and weekly samples were collected for further laboratorial analysis to confirm the absence of any residue (Bursa Uludag University, Bursa, Türkiye). Starting on d 7 of the study, all calves were offered a starter feed and wheat straw (93:7 ratio, respectively, DM basis) for ad libitum consumption (Table 1), whereas fresh and clean water were available at all times for ad libitum intake throughout the experimental period. Moreover, from d 7 to weaning (d 63), the calf starter and wheat straw were mixed and offered in plastic feed containers to the calves every morning (0900 h) after the morning whole milk feeding.

Sampling and Analysis

Daily DMI. Daily DMI was calculated based on daily consumed calf starter, wheat straw, and DM of milk. Feed samples were collected weekly and pooled for nutritional composition, including DM, ash, OM, CP, starch, and ether extract following methodologies previously described and validated (AOAC, 1990). Neutral detergent fiber concentration was determined according to Van Soest and Robertson (1979), whereas

ADF and ADL were calculated following procedures described by Goering and Van Soest (1970).

BW Measurements. Individual BW was assessed weekly from birth (d 0) to weaning (d 63). All BW measurements were performed using a digital scale (sensitivity = 5 g) that had been previously checked, certified, and validated. Based on the BW measurements, weekly and overall ADG was determined for animals receiving CON and DFM. Moreover, feed conversion ratio (**FCR**) was determined by dividing weekly feed intake and ADG (mixture of starter and wheat straw, as well as milk).

Adverse Health Observations. Health status of the calves was performed daily during the entire experimental period. For diarrhea assessment, observations were performed on a daily basis at 0830 h during the first 3 wk of the study, whereas weekly evaluations were performed from d 21 to weaning. To determine the proportion of animals presenting diarrhea, the following fecal scores were used: score 1 = watery (diarrhea), score 2 = soft with an indistinct format, score 3 = soft with a defined and limited format, score 4 = hard with a well-defined format, and 5 = hard "pellet" feces. Animals diagnosed with score 1 (diarrhea) were also observed for the number of days presenting the diarrhea score and a standard antibiotic treatment consisting of amoxicillin-clavulanic acid + enrofloxacin was used for all animals. Concomitantly with the fecal collection, individual rectal temperature was also performed in the first 3 wk of the trial with an electronic thermometer (Kruuse Premium Digital Thermometer, Jørgen Kruuse A/S, Langeskov, Denmark).

Fecal Analysis. In the first 3 wk of the trial, fecal samples were manually collected directly from the rectum of each calf for daily fecal pH determination (Verlinden et al., 2006). Approximately 5 g of fresh feces was collected and fecal pH determined using an electronic pH meter (PT-10, Sartorius Lab Instruments GmbH and Co. KG, Göttingen, Germany) after mixing with 15 mL of distilled water in a 50-mL beaker.

On d 33 and at weaning, fecal samples were taken manually and directly from the rectum of each animal approximately at 4 h after the morning feeding for fecal VFA analysis and a possible effect of DFM supplementation on gastrointestinal fermentation profile of preweaning dairy calves, as challenges might lead to changes in fecal VFA concentration (Patra and Kar, 2021). Approximately 2 g of fresh feces were placed in plastic tubes, acidified with 2 mL of 25% metaphosphoric acid, and diluted with 6 mL of distilled water (Flickinger et al., 2003). The mixture was centrifuged at 25,000 × *g* (Eba-21, Hettich GmbH & Co. KG, Tuttlingen, Germany) for 20 min at 4°C. Supernatants were stored frozen at −20°C until further laboratorial analysis. Before analysis, supernatants were kept at room temperature until dissolved and supernatants were centrifuged at 13,000 × *g* for 10 min at 4°C. One milliliter was taken and placed in vials. The VFA concentrations were determined by placing a gas chromatograph (Hewlett Packard Agilent Technologies 6890 N Network GC System, Series CN10447002, Beijing, China). A column (6- × 2-mm i.d. glass) was packed with 10% SP-1200/1% H_3PO_4 80/100 Chromosorb WAW (Supelco, Bellefonte, PA). Carrier gas (He) flow was 40 mL/min, column temperature was 130°C, and detector temperature was 175°C. The detection was carried out by flame ionization.

At weaning, an additional fecal sample was collected from 15 calves/treatment group, randomly selected, for microbiological analysis. For the isolation and enumeration of fecal microflora, approximately 5 g of fresh fecal samples were collected, aseptically transferred into a sterile stomacher bag, and homogenized with 45 mL of saline peptone water (Merck KGaA, Darmstadt, Germany) in a Seward Stomacher 80 Lab System for 2 min. Then, serial 10-fold dilutions were performed in the saline peptone water and plated in duplicates onto relevant selective media.

De Man, Rogosa, and Sharpe (Merck KGaA) agar was used for isolation and enumeration of *Lactobacillus* spp. following 3 d of incubation at 35°C under 5% CO_2 (Anaerobik Jar and Anaerocult; Merck KGaA). *Bifidobacterium* spp. were grown on *Bifidobacterium* selective medium agar (BSM agar #88517; Sigma Aldrich, Darmstadt, Germany) and the plates incubated at 37°C for 48 to 72 h under anaerobic conditions with gas-generating sachet (Anaerobik Jar and Anaerocult; Merck KGaA). For total coliform isolation and enumeration, samples were grown on violet-red bile lactose agar (#1.01406; Sigma Aldrich) using the "pour" plate technique and plates with 30 to 300 colonies used for enumeration following 24 to 48 h of incubation at 37°C. After incubation, typical colonies (red colonies with halos) were inoculated in Lactose Broth (#1.07661; Merck KGaA) using a Durham tube at 44°C for 24 h. After incubation, acid and gas formation from positive colonies were confirmed to be *Escherichia coli* using the indole, methyl red, Voges-Proskauer tests, where indole (+), methyl red (+), Voges-Proskauer (−), and citrate (−) indicated the presence of *Esh. coli* type-1 in the fecal samples (Cetin et al., 2016).

Last, *Eimeria* spp. counts were performed on fecal samples collected on d 21, 42, and 63 of the study and analyzed using a modified McMaster methodology. Four grams of feces were mixed into 56 mL of flotation liquid and filtered. A sample of the supernatant was taken with the help of a Pasteur pipette and dropped in a McMaster slide, which was kept at room temperature

for 3 to 5 min to allow the oocysts to float. The counting of oocysts in both compartments of the McMaster slide was taken and the oocyst amount per gram of feces was determined by using the formula described by others (Nansen et al., 1981; Ballweber et al., 2014). If the number of oocysts was considered high for the historic values of the research center, it was determined whether the *Eimeria* spp. would be considered pathogenic or not, given that oocysts of many *Eimeria* spp. acquire morphological features that aids into the differentiation of the species only after its sporulation. For this reason, in cases where species identification is required, sporulation of oocysts is used following techniques previously described in the literature (Levine, 1985).

For the *Cryptosporidium* analysis, fecal samples were taken from calves by rectal palpation and fecal smears, which were prepared with carbol fuchsin, were examined under light microscopy. Then, fecal scores were evaluated as 5 in stool samples (Medema et al., 2001, Constable et al., 2017) in which, oocyst counts were evaluated in stool samples under the microscope (Kuczynska and Shelton, 1999).

Statistical Analysis

All statistical analyses were performed considering the calf as the experimental unit and using the SAS Statistical Software (version 9.4; SAS Inc., Cary, NC). All the continuous variables (performance, fecal pH, fecal VFA, rectal temperature, blood variables, and microbiological counts) were analyzed using the MIXED procedure of SAS. The models contained treatment, day, or week, and the resulting interaction in the fixed effects, calf as the random variable, and calf (treatment) used as subject for the repeated statement. Moreover, the covariance structure currently tested included the compound symmetry and first-order autoregressive, whereas the latter was chosen as it provided the lowest Akaike information criterion. Results were reported as least squares means, whereas the microbiological data were log-transformed and reported as $\log_{10}$ cfu per gram of feces.

Diarrhea occurrence (fecal score = 1) was analyzed using the LOGISTIC procedure of SAS and the equation generated to calculate the odds ratio using the CON group as the reference. For protozoa occurrence (binomial; presence or absence), the GLIMMIX procedure of SAS was used, considering calf as the random variable, and treatment and week as fixed effects in the model statement.

For all the data, significance was set at $P \leq 0.05$ and tendencies were denoted if $P > 0.05$ and $P \leq 0.10$. Moreover, results are reported according to the main treatments effects if no interactions were determined to be significant.

RESULTS

Performance Results

A treatment × week interaction was observed for BW ($P < 0.01$), as DFM-supplemented calves were heavier than CON cohorts on d 56 (+ 4.7 kg) and at weaning on d 63 (+ 4.8 kg; $P \leq 0.01$; Figure 1A), whereas the same result tended to be observed on d 42 ($P = 0.10$). A similar interaction was observed for ADG, with greater ADG for DFM-supplemented calves from d 35 – 42 and from d 49 to 56 ($P \leq 0.04$; Figure 1B), and a tendency for greater ADG from d 7 to 14 ($P = 0.06$). Calves supplemented with DFM also had a greater total feed (milk, starter, and alfalfa hay mixture) intake (treatment × day interaction; $P < 0.01$) from d 42 to 49, 49 to 56, and 56 to weaning ($P \leq 0.04$; Figure 1C) and a lower mean FCR ($P = 0.03$) when compared with CON calves (Table 2).

Diarrhea, Microbiological, and Parasitological Results

Diarrhea cases were observed only in the first 2 wk of the present study and DFM-supplemented calves tended to have a lower occurrence when compared with CON calves ($P = 0.10$; Table 3). Conversely, no treatment effects were noted on fecal pH ($P = 0.44$), total or individual fecal VFA ($P \geq 0.17$), or microbiological counts ($P \geq 0.34$), but rectal temperature was lower for calves supplemented with DFM ($P = 0.04$; Table 4). Moreover, no treatment × day interactions were reported for the VFA data ($P \geq 0.34$; Table 4).

At weaning and regardless of treatment, *Eimeria* spp. and *Cryptosporidium* spp. were not detected on fecal samples collected from the animals. However, treatment × day interactions were observed for the occurrence and counts of *Eimeria* spp. ($P \leq 0.04$; Table 5), as DFM-supplemented calves tended to have a reduced number of positive observations for *Eimeria* spp. on d 42 of the study versus CON ($P = 0.08$). Moreover, a significant reduction in positive animals from d 21 to 42 was observed in DFM-supplemented calves ($P < 0.01$), but not in CON calves ($P = 1.00$). For *Cryptosporidium* spp., no occurrence was observed on d 42 of the study, so main treatment effects were analyzed only for fecal samples collected on d 21 of the study. No treatment effects were observed on occurrence of *Cryptosporidium* spp. (%; $P = 0.40$), but DFM-supplemented calves had a greater oocyst per gram versus CON ($P = 0.04$; Table 5).

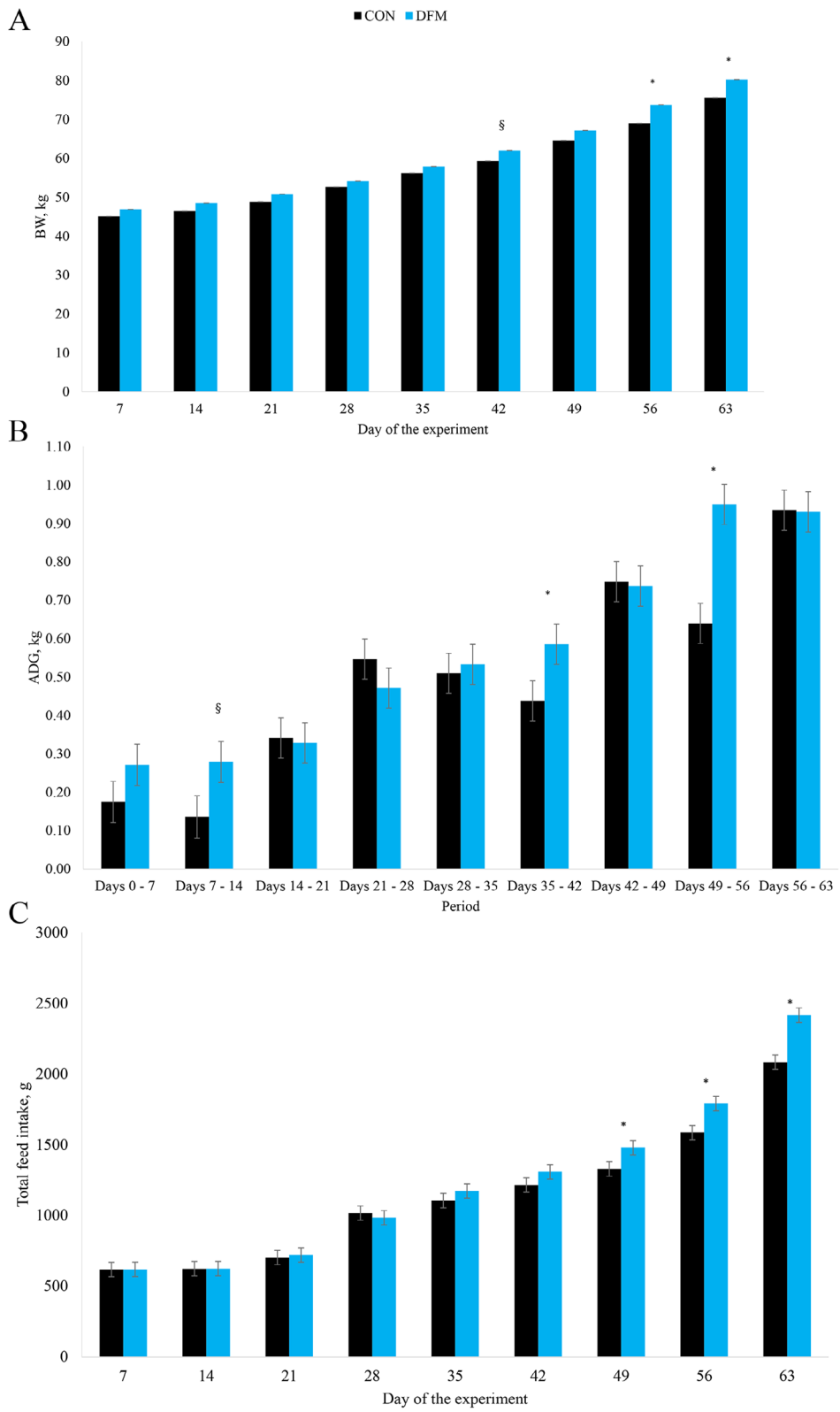


Figure 1. Weekly BW (A), ADG (B), and total feed (milk, starter, and alfalfa hay) intake (C) measurements of preweaning dairy calves supplemented (DFM; n = 21) or not (CON; n = 21) with a direct-fed microbial containing *Enterococcus faecium* 669 (Lactiferm; Chr. Hansen A/S; Hørsholm, Denmark) in the whole milk. A treatment × week interaction was observed herein ($P < 0.01$). * denotes significance at $P \leq 0.05$, and § denotes a tendency ($0.05 < P \leq 0.10$). Error bars are SEM.

Table 2. Performance results of preweaning dairy calves supplemented (DFM; n = 21) or not (CON; n = 21) with a direct-fed microbial containing *Enterococcus faecium* 669 (Lactiferm; Chr. Hansen A/S; Hørsholm, Denmark) in the whole milk[1]

	Treatment			*P*-value[2]		
Item	CON	DFM	SEM	T	D	T × D
BW, kg	57.5	60.1	1.05	0.08	<0.0001	<0.01
ADG, kg/d	0.496	0.565	0.0187	0.04	<0.0001	0.01
Feed intake, kg/d	1.14	1.24	0.031	0.04	<0.0001	<0.001
Feed conversion ratio, kg/kg	2.61	2.19	0.098	0.04	<0.0001	0.25

[1]Whole milk was used as the carrier for the direct-fed microbial for the entire 63-d experimental period.
[2]T = treatment effect; D = day effect; T × D = treatment × day interaction.

DISCUSSION

Direct-fed microbials (or probiotics), per definition, must bring health benefits to the host when offered in adequate amounts (FAO and WHO, 2001; Markowiak and Śliżewska, 2018). In this classical definition, performance improvements are not clearly stated, but it is well known and reported by other research groups that healthier dairy calves and cows will be more productive in the short, medium, and long term (Heinrichs and Heinrichs, 2011; Soberon et al., 2012; Carvalho et al., 2019). Hence, DFM that support health and performance of the dairy cow herd are desired and should be further studied to promote welfare and performance of the production systems. Therefore, this study was conducted to evaluate the effects of a DFM containing *Ent. faecium* 669 on health, performance, parasitological, microbiological, and hematological responses of preweaning Holstein dairy calves.

Enterococcus faecium is a gram-positive, lactic acid producer that has been used for DFM in several commercial products for cattle (Shridhar et al., 2022). In ruminants, supplementation of *Ent. faecium* alone or in combination with other DFM types has been improving rumen health, DMI, nutrient digestibility, and milk yield of dairy cattle, as well as feed efficiency of feedlot cattle (Nocek et al., 2003; Nocek and Kautz, 2006; Chiquette et al., 2012; Azzaz et al., 2022; Dias et al., 2022). In Europe, the Food Safety Authority has issued a positive opinion on *Ent. faecium* 669, approving its use in calves up to 6 mo of age (EFSA, 2012). In the United States, the genus Enterococci has not been granted the "generally recognized as safe status" (Franz et al., 2011), but few *Enterococcus* strains, including *Ent. faecium* 669, have been considered safe as they do not carry any major virulence genes (Shridhar et al., 2022), and hence have been used as DFM supplements to support the health of humans and livestock animals (Serio et al., 2010). As an example, in *Esh. coli*-challenged broiler chickens, *Ent. faecium* 669 supplementation improved jejunal villus height, villus height to crypt depth ratio, gene expression of tight junction proteins and mucin (MUC-2), as well as improved chicken performance and survival rates (Huang et al., 2019).

In the present study, preweaning supplementation of dairy calves with *Ent. faecium* 669 in the whole milk significantly resulted in heavier calves at weaning (+4.8 kg or 6.4%) and improved overall ADG (+69 g or 13.9%), feed intake (+90 g), and feed conversion ratio (−420 g of feed/kg gain or 16.1%). Recently, Cappellozza et al. (2023) demonstrated that *Ent. faecium* 669 inclusion into the whole milk also improved 56- and 63-d performance of preweaning dairy calves, with improvements on weaning BW and ADG ranging from 7.8% to 4.7% and 16.7% to 9.7%, respectively. Moreover, corroborating the current results, the latter authors also reported a similar positive trend on diarrhea occurrence for calves offered *Ent. faecium* 669 (Cappellozza et al., 2023). In the present experiment, feeding DFM also benefited ADG on wk 2 of the study, which agrees with the occurrences of diarrhea. So, DFM feeding supported the health and more specifically, gastrointestinal health, of

Table 3. Effects of treatment and week on diarrhea occurrence, 95% CI, and odds ratio analysis of preweaning dairy calves supplemented (DFM; n = 21) or not (CON; n = 21) with a direct-fed microbial containing *Enterococcus faecium* 669 (Lactiferm; Chr. Hansen A/S; Hørsholm, Denmark) in the whole milk[1]

Item	Estimate	SEM	*P*-value	Odds ratio[2]	95% CI
Intercept	0.152	1.0366	0.88	—	—
Treatment	−0.814	0.4925	0.10	0.443	0.169–1.163

[1]Whole milk was used as the carrier for the direct-fed microbial for the entire 63-d experimental period.
[2]Analysis for odds ratio using CON as the reference treatment.

Table 4. Fecal pH, rectal temperature, and fecal microbiological analysis of preweaning dairy calves supplemented (DFM; n = 21) or not (CON; n = 21) with a direct-fed microbial containing *Enterococcus faecium* 669 (Lactiferm; Chr. Hansen A/S; Hørsholm, Denmark) in the whole milk[1]

	Treatment			*P*-value[2]		
Item	CON	DFM	SEM	T	D	T × D
Fecal pH	6.88	6.83	0.038	0.44	—	—
Rectal temperature, °C	38.7	38.5	0.04	0.04	—	—
Fecal VFA,[3] mmol/L						
Acetate	17.4	18.6	0.78	0.31	0.15	0.41
Propionate	5.54	6.17	0.317	0.17	<0.01	0.56
Isobutyrate	0.86	1.04	0.091	0.17	<0.01	0.97
Butyrate	4.13	4.67	0.299	0.20	<0.01	0.64
Isovalerate	2.44	2.88	0.295	0.29	<0.01	0.34
Valerate	2.12	2.22	0.193	0.71	0.24	0.50
Microbial counts,[4] $\log_{10}$ cfu/g of feces						
Coliforms	7.30	7.52	0.182	0.40	—	—
Escherichia coli	7.19	7.27	0.192	0.77	—	—
Lactobacillus spp.	7.38	7.61	0.171	0.34	—	—
Bifidobacterium spp.	6.15	6.04	0.246	0.75	—	—

[1]Whole milk was used as the carrier for the direct-fed microbial for the entire 63-d experimental period.
[2]T = treatment effect; D = day effect; T × D = treatment × day interaction.
[3]Samples collected on d 33 and at weaning.
[4]Samples collected at weaning only.

preweaning dairy calves and concomitantly benefited the performance of the herd, corroborating with others (Heinrichs and Heinrichs, 2011; Signorini et al., 2012). In a meta-analysis, Signorini et al. (2012) demonstrated that calves fed lactic acid-based DFM had a reduced risk of presenting diarrhea and this preventive effect was dependent on the supplement type, with greater effects observed when whole milk versus milk replacer was offered (Signorini et al., 2012). Previous studies in the literature have demonstrated that calves diagnosed with diarrhea had reduced overall preweaning performance that also impaired long-term dairy cow performance, by reducing milk yield and composition in the first and subsequent lactations (Heinrichs and Heinrichs, 2011). Additionally, these production impairments become even more pronounced in calves that were diagnosed with diarrhea and were treated with antimicrobials during the preweaning period (Soberon et al., 2012).

Gastrointestinal disorders, such as diarrhea, are multifactorial and are the most prevalent diseases in preweaning dairy calves, with roughly 21% of North American dairy calves being affected, and 76% of these cases treated with antimicrobials (USDA et al., 2018). Treatments and preventive measurements for diarrhea, such as antimicrobials and DFM, respectively, aim to decrease the load of total coliforms in the lower gastrointestinal tract and, thereby, prevent bacteremia by different mechanisms. Antimicrobials clean, from a nonspecific standpoint, commensal and potentially

Table 5. Occurrence and counts of *Eimeria* spp and *Crypstosporidium* spp. (in oocysts/g of feces) in preweaning dairy calves supplemented (DFM; n = 21) or not (CON; n = 21) with a direct-fed microbial containing *Enterococcus faecium* 669 (Lactiferm; Chr. Hansen A/S; Hørsholm, Denmark) in the whole milk[1]

	Treatment			*P*-value[2]		
Item	CON	DFM	SEM	T	D	T × D
Eimeria spp. occurrence, %				0.61	0.04	0.04
d 21	28.6 (6/21)[a]	42.9 (9/21)[a]	9.34			
d 42	28.6 (6/21)[a]	4.8 (1/21)[b]	9.34			
Eimeria spp. count, oocysts/g of feces				0.05	0.10	<0.001
d 21	350.0[b]	183.3[c]	71.56			
d 42	150.0[c]	700.0[a]	106.27			
Cryptosporidium spp. occurrence, %	23.8	31.0	6.02	0.40	—	—
Cryptosporidium spp. count, oocysts/g of feces	17.4	38.1	6.66	0.04	—	—

[a–c]Different letters denote differences at $P < 0.05$ level.
[1]Whole milk was used as the carrier for the direct-fed microbial for the entire 63-d experimental period.
[2]T = treatment effect; D = day effect; T × D = treatment × day interaction.

harmful bacteria (Smith, 2015), whereas DFM support the population of commensal bacteria and competitively excluding these undesired bacterial species (Krehbiel et al., 2003; McAllister et al., 2011). In the present study, no differences were observed on total coliforms and *Esh. coli* counts from fecal samples collected at weaning. So, one may speculate that the sampling period adopted herein (1) was not adequate to find potential differences on pathogenic bacteria and (2) also did not take into consideration the analysis of other important pathogenic bacteria, such as *Salmonella* spp. and *Clostridium* spp. (Lucey et al., 2021), that could be involved in 7- or 14-d cases of diarrhea in calves.

Another interesting finding of the present study was that DFM supplementation reduced mean rectal temperature of preweaning calves by 0.2°C. Nonetheless, the rectal temperature range reported herein is considered adequate for preweaning calves (Hill et al., 2016) and one potential pitfall of the present study was that we could not measure the temperature constantly (i.e., by using rectal data loggers) in the first weeks of the trial to evaluate potential daily fluctuations in rectal temperature and how it might correlate with the health status of the animals. Others have also reported that different feeding strategies, such as prebiotics, during the preweaning period did not affect fecal pH, whereas fecal VFA might be dependent on the sampling schedule or feed additive being used (Vadopalas et al., 2021).

Coccidiosis is a self-limiting protozoal disease mainly caused by coccidia of the genus Eimeria (Kemp et al., 2013). More specifically, *Eimeria* spp. are generally well recognized as gastrointestinal parasites in poultry and ruminants, leading to enteritis with occurrences of diarrhea, dehydration, and weight loss. Feeding a DFM mixture containing *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* reduced lower duodenal and jejunal lesion scores and improved performance of *Eimeria*-challenged broilers, suggesting that lactic acid-producing DFM bacteria can support the lower gastrointestinal tract health of birds (Giannenas et al., 2014; Ritzi et al., 2014). To the best of our knowledge, few studies have evaluated the effects of DFM on *Eimeria* spp. occurrence and counts. Recently, in vitro invasion of *Eimeria tenella* in bovine kidney cells was inhibited by *Ent. faecium* (Hessenberger et al., 2016). Our results indicate a potential positive effect of *Ent. faecium* 669 supplementation on reducing the occurrence of *Eimeria* spp. in the feces of preweaning dairy calves, but caution should be taken when interpreting these results as the number of calves enrolled in the trial was small and even the total counts (in oocysts/g of feces) may be lower than often seen in clinical cases. Moreover, the effects, if any, of DFM supplementation to calves on *Cryptosporidium* spp. have been inconclusive and variable at this point. Recently, Lucey et al. (2021) demonstrated that feeding a *Bacillus subtilis*-based DFM to preweaning dairy calves reduced *Cryptosporidium* spp. shedding on d 14 versus a nonsupplemented control, but no differences were observed when calves were 21 d old. Although a treatment effect on oocyst per gram of feces was observed in the present study, numbers were considered lower than previous research reports (Lucey et al., 2021) and caution should be taken when evaluating such results, as aforementioned. One may speculate that the whole improvements of feeding a DFM, including the lactic acid-producing bacteria *Ent. faecium* 669, on lower gastrointestinal tract health are due to a complex and multifactorial benefit, such as direct inhibition of pathogens by the action of bacteriocins and modulating the pH of the medium (Kung et al., 2003; Muck et al., 2018), but also by indirect mechanisms, such as competitive inhibition, modulation of the gut microbiota favoring the growth of commensal bacteria in the host (Pajarillo et al., 2015), modulation of the immune system and its response (Qadis et al., 2014a,b), support of intestinal barrier integrity, and stimulation of mucin production that alleviates potential damaging effects of pathogenic bacteria or stressors. Nonetheless, additional studies are warranted to understand the supportive mechanisms of *Ent. faecium* 669 against protozoal agents.

In summary, supplementing a direct-fed microbial based on *Ent. faecium* 669 improved overall performance of preweaning Holstein dairy calves, while also reducing diarrhea occurrence in the first 2 wk of life, rectal temperature, and *Eimeria* spp. detections from d 21 to 42. Additional studies are warranted to elucidate specific and novel modes of action, if any, of *Ent. faecium* 669 in preweaning dairy calves.

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