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Short Communication
Animal Nutrition and Farm Systems

Effects of supplementing a *Bacillus*-based direct-fed microbial on performance, nutrient digestibility, rumen fermentation characteristics, and metabolic responses of lactating dairy cows

Bruno I. Cappellozza,[1] Dannylo O. Sousa,[2] Christine Alveblad,[3] Oscar Queiroz,[1] Jens N. Joergensen,[1] and Bengt-Ove Rustas[3]*

Graphical Abstract

In dairy production systems, improvements on milk yield and milk production efficiency are imperative to maximize current challenges

Bacillus-based direct-fed microbial (**DFM**) might be a feasible alternative, given its health benefits and enzyme-production ability

Feeding the DFM improved milk yield and milk production efficiency of lactating dairy cows

Summary

Direct-fed microbials (DFM) have been shown to support the health and performance of the dairy cow herd, but differences in these results might be seen. To date, few studies have evaluated the effects of adding *Bacillus* spp. (*B. licheniformis* and *B. subtilis*) on performance and metabolic responses of lactating dairy cows. Overall, feeding a *Bacillus*-based DFM resulted in greater milk yield, lactose yield, total solids yield, and milk production efficiency, and tended to increase protein yield and energy-corrected milk production efficiency. Additionally, cows fed DFM had a greater mean insulin-like growth factor-I (IGF-I) concentration. In summary, feeding a *Bacillus*-based DFM improved the performance and metabolic responses of lactating dairy cows.

Highlights

- DFM are feed additives that, when fed in adequate amounts, support adequate health, nutrient digestibility, and performance of lactating dairy cows.
- Feeding a *Bacillus*-based DFM for 84 days improved milk yield, milk production efficiency, lactose yield, and total solids yield of lactating dairy cows.
- Cows fed DFM had a greater mean IGF-I concentration during the study.

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Abstract: This experiment was conducted to evaluate the effects of feeding a *Bacillus*-based direct-fed microbial (DFM) on performance, nutrient digestibility, rumen fermentation, and metabolic response of lactating dairy cows. Sixty-eight lactating (50 ± 6 d in milk) Holstein-Friesian (n = 20) and Swedish Red (n = 48) cows were enrolled to a 15-wk experiment. Cows were blocked by breed, lactation number, and days in milk and, within blocks, assigned to 1 of the 2 treatments: (1) basal partial mixed ration (PMR) without DFM (n = 34; CON) or (2) basal PMR with the addition of 3 g/head per day of a DFM containing *Bacillus licheniformis* and *Bacillus subtilis* (n = 34; DFM). The DFM was mixed in a protein pellet, whereas the CON group was fed the same pellet without DFM (1 kg/cow per day). The PMR contained 53% clover grass silage and 47% compound feed plus 3 kg of a concentrate (dry matter basis) offered during milking. Milk yield and production efficiency were recorded daily, whereas milk samples were collected for 24 h every second week of the study for milk composition. During the experimental period, fecal, rumen fluid, and blood samples were collected from each cow for apparent nutrient digestibility, rumen fermentation, and metabolic responses, respectively. All data were analyzed using the MIXED procedure of SAS. No treatment effects were observed on cows final body weight and daily dry matter intake. However, cows fed DFM had a greater milk yield, milk production efficiency, lactose and total solids yield, and also tended to have a greater energy-corrected milk production efficiency and milk protein yield. No significant differences were observed on nutrient digestibility and total volatile fatty acids, but molar proportion of acetate was greater for cows fed DFM. In contrast, molar proportion of propionate was greater and butyrate tended to be greater for CON. Cows fed DFM had greater mean plasma insulin-like growth factor-I (IGF-I), but no differences were observed for plasma glucose and insulin. In summary, supplementing a *Bacillus*-based DFM benefited productive responses of lactating dairy cows, while also modulating rumen fermentation and serum IGF-I.

Over the last years, dairy producers have been challenged to comply and adhere to environmental guidelines (Britt et al., 2018), while also maintaining productivity and profitability of the dairy operation in periods of increasing feed prices. In this challenging scenario, dairy producers look for technologies that (1) promote milk yield and (2) optimize milk production efficiency, thus supporting the profitability of the operation (Thomas et al., 2023).

Direct-fed microbials (**DFM**) are live bacteria that support the health (Markowiak and Śliżewska, 2018) and performance of dairy cows, including milk yield, composition, and milk production efficiency (Nocek and Kautz, 2006; Valldecabres et al., 2022). Several bacterial species have been fed as DFM for cattle, including *Lactobacillus* spp., *Enterococcus* spp., and *Bacillus* spp. Bacilli have been gaining attention among nutritionists, producers, and veterinarians for their (1) application in different feed supplements, surviving feed preparation (Bernardeau et al., 2017), (2) health-supportive effects (Segura et al., 2020), (3) support to the integrity of gut cells, and (4) improvements on nutrient digestibility (Pan et al., 2022) that, altogether, result in positive effects on milk production and efficiency of dairy cows (Oyebade et al., 2023). However, few data are available evaluating productive performance, in vivo nutrient digestibility, rumen fermentation characteristics, and metabolic responses of lactating dairy cows receiving a *Bacillus*-based DFM. Hence, we hypothesized that feeding a DFM containing *Bacillus* spp. would improve milk yield, composition, and production efficiency, as well as nutrient digestibility, rumen fermentation traits, and metabolic responses of lactating dairy cows. Therefore, our objective was to evaluate the effects of supplementing a *Bacillus*-based DFM on performance, nutrient digestibility, rumen fermentation traits, and metabolic responses of lactating Holstein-Friesian and Swedish Red cows.

This experiment was conducted from September 2021 to June 2022 at the Swedish Livestock Research Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden. All procedures were approved by the Uppsala Ethics Committee for Animal Research (Dnr. 5.8.18–06017/2022).

On d 0, 68 lactating (50 ± 6 DIM) Holstein-Friesian (n = 20; 6 primiparous and 14 multiparous) and Swedish Red (n = 48; 2 primiparous and 46 multiparous) cows were enrolled to a 15-wk

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experimental period, comprising 2 wk of adaptation to the pens, 1 wk for covariates, and 12 wk of individual data collection. Cows were fed a partial mixed ration (**PMR**) and on top of that offered 4 kg/d per cow of a protein-based pelleted concentrate. The PMR contained 53% grass-clover silage and 47% compound feed (DM basis), and the whole ration was formulated to meet or exceed the nutritional requirements of lactating dairy cows producing at least 40 kg of milk/d (Volden, 2011). Table 1 reports the composition and nutritional profile of the protein concentrate, compound feed, and clover grass silage used herein. Cows were blocked by breed, parity, and DIM, and within blocks, assigned to (1) PMR and protein concentrate (n = 34; **CON**) or (2) PMR and protein concentrate with the addition of 3 g/head per day of a DFM containing a mixture of *Bacillus licheniformis* and *Bacillus subtilis* (Bovacillus; 3.2×10^9 cfu/g; Chr. Hansen A/S, Hørsholm, Denmark; n = 34; **DFM**). The DFM was included into a portion of the protein-based pelleted concentrate and offered at 1 kg/cow per day to the DFM group, whereas CON cows were also fed 1 kg of the protein-based pellet daily.

All cows were fed the PMR in amounts to ensure ad libitum intake with at least 5% of daily feed refusal using feed bunkers with individual feeding recording (Biocontrol A/S, Rakkestad, Norway). The PMR was offered 5 times per day (0800, 1100, 1430, 1800, and 2200 h), and at each feeding event, a new feed batch was automatically prepared in a stationary mixer (Triolet T20, Trejon AB, Vännäsby, Sweden). Each morning, the bunkers were cleaned before the first feeding of the day. One kilogram of protein-based pellet concentrate, with or without DFM, was offered in separate automatic concentrate dispensers (FSC-400, DeLaval, Tumba, Sweden) placed in the resting area. During milking, in the automatic milking system (DeLaval), all cows enrolled in the trial were fed 3 kg of protein-based pellet concentrate/d (DM basis), without DFM.

Table 1. Composition and nutritional profile of silage and compound feed in the partial mixed ration and protein-based pellet concentrate fed separately to the animals during the present experiment[1,2]

Item	Silage + compound feed	Protein-based pellet concentrate
Ingredient, % DM		
Grass-clover silage	53.0	—
Heat-treated rapeseed meal	4.7	28.4
Rumen-protected soybean meal	—	13.5
Dried distillers grain	2.3	13.8
Barley	14.0	—
Wheat	8.5	13.0
Wheat bran	5.5	—
Beet pulp molasses	1.9	11.0
Palm kernel expeller	2.8	6.0
Rapeseed	0.5	5.0
Rapeseed cake	5.2	—
Vegetable fat	—	2.8
Rapeseed expeller	—	2.0
Soybean molasses	0.7	2.0
Soybean meal	—	1.0
Mineral-vitamin premix	1.0	1.5
Nutritional profile		
DM, %	58.7	89.1
CP, % DM	17.6	27.1
NDF, % DM	31.4	21.5
ADF, % DM	18.5	15.2
Ash, % DM	8.1	7.6
Crude fat, % DM	2.3	7.8
Starch, % DM	14.0	13.6

[1]Partial mixed ration (53% silage and 47% compound feed on a DM basis) and protein-based pellet concentrate offered daily.

[2]Protein-based pellet concentrate was offered separately at the automatic milking system.

Grass-clover silage was collected every week, frozen at −20°C, and pooled every 2 wk, whereas the compound feed, protein pellets, and mineral mix were sampled twice per week and pooled over a 4-wk period. Silage DM was determined after drying at 60°C for 18 h (Volden, 2011), whereas the DM content of all other feedstuffs was determined by drying at 103°C overnight. Ash content was determined by ignition at 550°C for 4 h, CP was analyzed by an automated Kjeldahl procedure (Foss, Hillerød, Denmark), and crude fat determined according to Commission Directive 98/64/EC (European Economic Community, 1998). Ash-free NDF was analyzed according to Chai and Udén (1998), and ADF determined according to Van Soest et al. (1991). All feed samples were analyzed for acid-insoluble ash (**AIA**) following Van Keulen and Young (1977).

On average, cows were milked 2.5 times per day and milk yield of the cows was recorded daily during the entire experiment (VMS, DeLaval) and, at milking, BW was recorded. Milk samples were collected for 24 h every second week of the experiment for milk fat, protein, lactose, and urea-N determination, whereas SCC used an LED flowcytometry methodology (Combiscope FTIR-300HP, Delta Instruments B.V., Drachten, the Netherlands). Milk composition was averaged by sampling week and milk fat, protein, lactose, and TS yields were determined by multiplying the weekly average milk yield with the concentration of milk fat, protein, lactose, and TS from the test day of each cow. Individual cow DMI was evaluated weekly and milk production efficiency was determined by dividing milk yield by DMI. Moreover, ECM (Sjaunja et al., 1990) was calculated using a published equation, whereas ECM production efficiency was determined by dividing ECM by DMI.

On wk 3 (covariate), 9, and 15 of the study and for 4 d within each week, 300 g of fecal sample was manually collected in the morning and in the afternoon from 38 cows (19 cows/treatment) for apparent nutrient digestibility analysis. Samples were stored at −20°C and after thawing, samples were thoroughly mixed, pooled within cow and week, freeze-dried, milled, and analyzed for DM, ash, CP, NDF, and AIA as described above. Fecal excretion was calculated from the total intake of AIA and fecal AIA content, assuming 100% AIA recovery (Van Keulen and Young, 1977). The apparent digestibility of DM, OM, CP, and NDF was calculated from estimated intake and excretion of each nutrient. Concurrently with the sampling for nutrient digestibility on wk 3 and 15, rumen fluid (n = 11 cows/treatment) and blood (n = 20 cows/treatment) samples were collected for 4 consecutive days and in the last day of the week, respectively. An oral-stomach probe containing a tube with a metal head acting as a sieve in the rumen and a manual pump with a 1-L glass container was used for rumen fluid collection. Approximately 1,000 mL of initially sampled ruminal fluid was discarded due to possible saliva contamination and, then, an additional 1,000 mL of ruminal fluid was collected. Subsamples were immediately frozen and stored at −20°C until analysis of VFA (Ericson and André, 2010). Blood samples were collected from the tail-head artery into commercial blood collection tubes containing

Table 2. Performance results of lactating dairy cows supplemented or not (CON; n = 34) with a *Bacillus*-based direct-fed microbial (DFM; n = 34)[1]

Item	Treatment		SEM	*P*-value
	CON	DFM		
BW, kg				
Initial	669.6	662.0	14.3	0.70
Final	690.4	683.3	14.5	0.73
DMI, kg/d	27.3	27.3	0.27	0.83
PMR	24.1	23.9	0.26	0.52
Pellet-based concentrate	3.2	3.3	0.05	0.04
Milk yield, kg/d	40.1	41.3	0.37	<0.01
Milk production efficiency, kg milk/kg DMI	1.48	1.52	0.019	0.02
ECM yield,[2] kg/d	41.8	42.7	0.57	0.11
ECM production efficiency, kg ECM/kg DMI	1.54	1.58	0.021	0.10
Yield, kg/d				
Protein	1.44	1.47	0.016	0.09
Fat	1.71	1.74	0.031	0.41
Lactose	1.84	1.92	0.043	0.03
TS	4.98	5.12	0.072	0.04
Milk composition, %				
Protein	3.59	3.56	0.021	0.15
Fat	4.29	4.21	0.062	0.22
Lactose	4.58	4.58	0.012	0.57
TS	12.5	12.3	0.08	0.16
MUN, mg/dL	39.1	38.4	0.86	0.35
SCC, × 1,000 cells/mL	271.3	250.8	11.24	0.53

[1]CON = basal partial mixed ration (PMR) offered to ensure ad libitum intake throughout the trial; DFM = CON diet with the addition of 3 g/head per day of a *Bacillus*-based DFM (Bovacillus, Chr. Hansen A/S, Hørsholm, Denmark).
[2]ECM = milk (kg) × [383 × (fat %) + 242 × (protein %) + 157 × (lactose %) + 20.7]/3,140 (Sjaunja et al., 1990).

lithium heparin (BD Vacutainer, 10 mL, Becton Dickinson, Franklin Lakes, NJ). Samples were placed immediately on ice, centrifuged (2,500 × *g* for 30 min, 4°C) for plasma harvest, and stored at −20°C. All samples were analyzed for plasma concentrations of glucose (#E-8140, R-Biopharm AG, Darmstadt, Germany), insulin (#10–1201–10, Mercodia, Uppsala, Sweden), and IGF-I (#E10, Mediagnost, Reutlingen, Germany). The intra-assay was 4.9% for glucose, 3.5% for insulin, and 3.2% for IGF-I.

The sample size was determined with the UBC Power Calculator (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html) using an α of 0.05 and power of 0.80 to detect 2.5% difference on milk yield. All data were analyzed using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC), the Satterthwaite approximation to determine the denominator df for the test of fixed effects, and block as random variable. Data obtained on wk 3 were used as covariates and production and nutrient digestibility data were analyzed using the repeated statement of SAS, and considering week as the repeated term, cow as the subject. The first-order autoregressive structure was chosen as it provided the lowest Akaike information criterion. Fixed effects included treatment, week, and treatment × week interaction. Somatic cell counts were log-transformed to meet the requirement of normal distribution assumptions and back-transformed for reporting in the manuscript. All data were reported as least squares means and covariate-adjusted to the values obtained on wk 3. Significance was set at $P \leq 0.05$, tendencies denoted if $0.05 < P \leq 0.10$, and results are reported according to the main effects if no interactions were significant ($P \leq 0.05$).

During the covariate period, no differences were observed for cow BW or for any of the productive and milk composition variables ($P \geq 0.30$; data not shown), but all covariates were significant ($P < 0.001$). For all the analyses, no significant treatment × week interactions were observed ($P \geq 0.07$) and, therefore, only main effects are presented and discussed. No treatment effects were observed on cow initial or final BW and daily total DMI ($P \geq 0.73$; Table 2). However, cows fed DFM had a greater concentrate intake, milk yield, milk production efficiency (as kg milk/kg DMI), lactose yield, and TS yield ($P \leq 0.04$), but also tended to have a greater ECM production efficiency and milk protein content ($P \leq 0.10$; Table 2) when compared with CON.

For nutrient digestibility, values obtained on wk 3 did not differ between treatments ($P \geq 0.30$; data not shown) and NDF was the only significant covariate ($P < 0.001$). No treatment effects were observed for DM, OM, CP, and NDF digestibility ($P \geq 0.13$; Table 3). Rumen and blood values obtained on wk 3 were not significant covariates and did not differ ($P > 0.07$; data not shown). No treatment effects were observed on total VFA ($P = 0.34$; 101.8 vs. 90.2 mmol/L for CON and DFM, respectively; SEM = 14.7), but molar proportion of acetate was greater for cows fed DFM ($P < 0.01$). On the other hand, molar proportion of propionate was greater ($P < 0.01$) and butyrate tended to be greater for CON ($P = 0.09$; Table 3). Apart from plasma IGF-I ($P = 0.03$), blood variables were not significant covariates ($P \geq 0.33$) and did not differ between treatments ($P \geq 0.18$; data not shown). Cows fed DFM had greater mean plasma IGF-I ($P = 0.05$), but no differences were observed on plasma glucose and insulin ($P \geq 0.43$; Table 3).

The main goal of the present experiment was to evaluate the effects of feeding a *Bacillus*-based DFM (*B. licheniformis* and *B. subtilis*) on performance, nutrient digestibility, and metabolic responses of lactating dairy cows fed a partial mixed ration. Direct-fed microbials have been used at an increasing rate in livestock

species, given their benefits in the health and performance of the herd (Cappellozza et al., 2023b), as well as being a potential replacement for antibiotics in ruminants. Moreover, previous research has reported the beneficial effects of bacilli in ruminants from all categories and ages (Kowalski et al., 2009; Sun et al., 2013; Lucey et al., 2021), with an additional advantage that these strains tolerate most, if not all, well-known challenges that a bacterium may face during feed preparation, including high temperature during pelleting, moisture, and the presence of minerals (Cappellozza et al., 2023a). In the present study, supplementing a *Bacillus*-based DFM for 84 d improved daily milk production by 1.2 kg/cow, milk lactose (+80 g) and TS (+140 g) yield, as well as milk production efficiency (+40 g milk/kg DMI), and tended to improve protein yield (+30 g) and ECM production efficiency (+40 g) versus nonsupplemented lactating cows. The tendency of improvements in milk protein and significant improvements on TS yield following DFM feeding could be related to the commensal effects of probiotics in the rumen environment and microbiota, stimulating the growth of beneficial microorganisms that enhanced the total amount of microbial protein produced and absorbed in the lower gastrointestinal tract (**GIT**; Nalla et al., 2022). Recently, Oyebade et al. (2023) reported a greater FCM yield in lactating dairy cows fed a DFM mixture containing *B. licheniformis* and *B. subtilis* and when compared with a nonsupplemented DFM group, greater milk fat yield and fat digestibility were also observed in bacilli-fed cows.

Contrary to our hypothesis, DFM feeding did not affect nutrient digestibility, but increased rumen acetate and lowered propionate proportions. In dairy cattle, feeding a DFM containing bacilli did not change the molar proportion of individual or total VFA, but increased fat digestibility when compared with a nonsupplemented group (Oyebade et al., 2023). Nonetheless, the increase in rumen acetate proportion without changes in milk fat (content or yield) or nutrient digestibility was unexpected, as the former is often associated with greater fiber digestibility and, therefore, milk fat content. Furthermore, the DFM used herein has been shown to promote in vitro DM and NDF digestibility in individual feedstuffs, but also in complete TMR collected from dairy farms in the United States (Pan et al., 2022; Cappellozza et al., 2023c). In low-producing dairy cows, Sun et al. (2013) reported that feeding *B. subtilis natto* increased milk yield, ECM, as well as yields of fat, protein, and lactose over a 70-d period, while also affecting DM and NDF digestibility and rumen fermentation traits. Nonetheless, differences in cow production status, DIM, breed, dietary nutrient composition, and profile among studies might also lead to the differences in the productive and milk composition responses of lactating dairy cows fed a *Bacillus*-based DFM (Krehbiel et al., 2003; Seo et al., 2010).

In the present study, mean plasma IGF-I concentration was higher for DFM-fed cows, but no significant differences were observed on mean plasma concentration of glucose and insulin, corroborating with previous studies in dairy cows (Oyebade et al., 2023). Circulating concentrations of IGF-I have been positively associated with the nutritional and healthy status of the dairy cattle herd (Beltman et al., 2020), so that healthier and more productive cows often have greater IGF-I concentrations than cows presenting an adverse health event or showing a lower milk production. Wathes et al. (2021) demonstrated that early-lactating cows with higher IGF-I concentrations also had grater milk protein yield, corroborating our results on IGF-I and milk protein yield. To the best of our knowledge, few studies have evaluated the effects of feeding a *Bacillus*-based DFM on IGF-I concentrations in lactating dairy cows. In beef calves, supplementing either *B. amyloliquefaciens* or *B. subtilis* improved FCR and increased blood IGF-I levels after 30 d (Du et al., 2018). Alternatively, postbiotic feeding has been shown to increase ruminal expression of genes related to VFA transport and hepatic expression of *IGF1* in newly weaned lambs (Izuddin et al., 2019). Altogether, these data suggest that a higher ruminal VFA uptake and microbiota modulation in the rumen and lower GIT could be mediating the improvements in performance of ruminants fed DFM (Nalla et al., 2022). In fact, the effects on milk lactose and TS yield, as well as the lack of effects on serum glucose and insulin, even though differences on rumen propionate have been noted, may support this rationale, but additional studies are warranted to evaluate such assumptions.

Table 3. Nutrient digestibility, rumen fermentation traits, and metabolic responses of lactating dairy cows supplemented or not (CON; n = 34) with a *Bacillus*-based direct-fed microbial (DFM; n = 34)[1]

	Treatment			
Item	CON	DFM	SEM	*P*-value
Nutrient digestibility, %				
DM	73.6	72.8	0.45	0.20
OM	75.3	74.5	0.43	0.16
CP	69.4	68.9	0.61	0.52
NDF	68.5	67.0	0.65	0.13
Molar proportion, m*M*/100 m*M*				
Acetate	60.4	64.4	1.55	<0.01
Propionate	25.0	22.4	0.86	<0.01
Butyrate	13.0	11.6	0.87	0.09
Valerate	3.64	3.53	0.215	0.53
Plasma variable				
Glucose, mmol/L	4.28	4.14	0.228	0.58
Insulin, µg/L	0.452	0.363	0.1096	0.43
IGF-I, ng/mL	193.1	220.6	10.91	0.05

[1]CON = basal partial mixed ration (PMR) offered to ensure ad libitum intake throughout the trial; DFM = CON diet with the addition of 3 g/head per day of a *Bacillus*-based DFM (Bovacillus, Chr. Hansen A/S, Hørsholm, Denmark).

As reported by Thomas et al. (2023), feed efficiency is a comprehensive indicator of dairy cattle performance, ultimately driving a dairy farm's profitability. Therefore, technologies that promote milk production efficiency are more sustainable and more likely to support the profitability of dairy operations, as more kilograms of milk are obtained per kilogram of feed that was consumed by the herd. Supporting our results, Valldecabres et al. (2022) showed improvements on milk production efficiency in lactating dairy cows fed a 4-strain DFM versus non-DFM-supplemented group or cows fed a 2-strain DFM throughout lactation. Therefore, it can be speculated that the improvements on milk production efficiency, as reported herein and by others, could be multifactorial, as supporting both the rumen and lower GIT environment can benefit milk yield, milk composition, and milk production efficiency.

In summary, feeding a *Bacillus*-based DFM to lactating dairy cows improved milk yield and milk production efficiency, while also affecting milk composition, rumen fermentation, and plasma IGF-I. Additional studies are warranted to understand the associations, if any, between the rumen and lower GIT environment with the fermentation traits and metabolic responses of lactating dairy cows under different productive stages and management when offered bacteria-based DFM.

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