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ORIGINAL ARTICLE

Effect of supplemental Bacillus cultures on rumen fermentation and milk yield in Chinese Holstein cows

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Summary

Two experiments were conducted to study the effect of supplemental 100 g/day of live Bacillus cultures (2×10^{11} cell of *Bacillus subtilis* and *Bacillus licheniformis*) on rumen fermentation as well as milk yield and composition in Chinese Holstein cows. In experiment 1, investigating 3×10 cows, milk yield and milk protein were increased by using *B. licheniformis* ($p < 0.05$) in comparison with an unsupplemented group and the *B. subtilis* group. Body weight was not significantly affected by Bacillus culture supplementation ($p > 0.05$). Percentage of milk fat and lactose was not significantly different between treatments ($p > 0.05$). But milk protein increased with *B. licheniformis* supplementation ($p < 0.05$). In experiment 2, carried out with three non-lactating ruminally and duodenally fistulated cows, results showed that *B. licheniformis* supplementation increased microbial crude protein flow into duodenum ($p < 0.05$) and decreased the ammonia nitrogen concentration in ruminal fluid at 0.5 h, 1 h, 3 h, 6 h after morning feeding ($p < 0.05$). *Bacillus licheniformis* supplementation increased total VFA and acetate concentration in ruminal fluid at 0.5 h, 1 h, 3 h, 6 h after morning feeding ($p < 0.05$). *Bacillus subtilis* had no significant effect on rumen fermentation characteristics, duodenal microbial N flow and ruminal apparent nutrient digestibility ($p > 0.05$). *Bacillus licheniformis* increased ruminal apparent nutrient digestibility of neutral detergent fibre, acid detergent fibre, and organic matter ($p < 0.05$).

Introduction

The term 'direct-fed microbial (DFM)' has been defined as 'a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989; Krehbiel et al., 2003). Concern regarding the use of antibiotics and other growth stimulants in the animal feed industry has increased in recent years. There has been increasing emphasis placed on disease prevention as a means of reducing the use of antibiotics. A definitive mode of action for bacterial or fungal DFM has not been established, although a variety of mechanisms have been suggested. These include the modification of rumen or lower gut microbial populations, alteration of rumen fermentation patterns, increased intestinal nutrient flow, improved diet digestibility and immune system modulation (Yoon and Stern, 1995; Krehbiel et al., 2003). The effect of DFM supplementation on cow performance or rumen fermentation has been reviewed by several authors (Martin and Nisbet, 1992; Jouany, 1994; Newbold, 1995; Nocek and Kautz, 2006). Although DFM supplementation has improved milk production, component yield, feed efficiency and health, animal response to DFM have been inconsistent. In

addition, results of DFM studies conducted with dairy cattle are difficult to compare because of the many different organisms, strains of organisms, and combinations of multiple organisms that have been supplemented. Other differences among studies include the DFM inclusion level in the diet, diet composition, feed intake and feeding frequency, along with animal factors such as age, physiological stage, health and stress status (Wagner et al., 1990). Variable response to feeding bacterial DFM in ruminant production systems emphasizes the need for greater understanding of underlying mechanisms. The objective of this study was to determine the effect of added Bacillus culture on milk yield and composition and on apparent total tract nutrient digestibility and rumen fermentation of Chinese Holstein cows in early lactation.

Materials and methods

Substrates and additives

Bacillus cultures were prepared by liquid culture and solid fermentation. Microbes were provided by China general microbiological culture collection centre, Institute of Microbiology, Chinese Academy of Sciences. In detail, these were *B. subtilis* (strain number 1.1086) and *B. licheniformis* (strain number 1.183). Both Bacillus species were reactivated and maintained on nutrient agar slants. The freeze-dried microbes were suspended using sterilized 0.9% NaCl solution. The maintenance medium contained in g/l: peptone 10, beef extract 3, NaCl 5, agar 15, $MnSO_4$ 0.0308, KH_2PO_4 0.5, K_2HPO_4 0.3, $CaCO_3$ 0.01, distilled water 1 litre. The medium was autoclaved at 121 °C for 20 mins, cooled to room temperature.

The liquid state medium was comprised the following: glucose 15 g, soluble starch 1 g, $MnSO_4$ 0.0308 g, KH_2PO_4 0.5g, K_2HPO_4 0.3 g, $CaCO_3$ 0.1 g, 1 l malt wort. The medium in shake flask was autoclaved at 121 °C for 20 mins. After cooling to the room temperature, *B. licheniformis* and *B. subtilis* were inoculated into the medium and kept at 37 °C, $4.0 \times g$ water bath for 24 h respectively.

The solid state medium was comprised the following: maize starch 5%, soybean powder 5%, wheat bran 30% and 60% water. The medium was autoclaved at 121 °C for 60 mins. After cooling, the liquid medium was inoculated into solid medium and was kept at 37 °C in a carbon dioxide incubator (ULT-1386-3, USA) for 48 h. The inoculum ratio was 10% (wt/wt); the inoculation was performed using sterilized pipette. Then the cultures were lyophilized and kept in refrigerator until use.

For colony counting of Bacillus, 1 g of each culture was 10-fold serially diluted (10^3–10^{10}) in sterile water. Enumeration was carried out using the pour plate technique (Dave and Shan, 1996). Plates in duplicate were incubated anaerobically at 37 °C for 72 h using a gas mixture of 10% $C0_2$, 5% H_2 and 85% N_2. Plates containing 15–250 colonies were counted, enumerated and recorded as colony forming units per gram of culture. All the countings were repeated thrice. The results presented are mean values of three replicates. After enumeration, cultures prepared using the above method contained 2×10^9 live cells per gram culture.

Animals, diet and management

Two experiments were conducted in this study. In experiment 1, 30 Chinese Holstein cows were selected on the basis of age, days in milk (approximately 50.5 days) and mean daily 2-week pre-trial milk yield (approximately 24.5 kg/day). The cows were randomly assigned to three treatment groups to determine the effect of supplemental *B. subtilis* and *B. licheniformis* individually in diet in comparison with an unsupplemented control group. The experimental animals were kept in a tie-stall barn. The cows in the treatment groups received 100 g Bacillus culture twice daily as a top-dress. Animals first received a portion of the ration with the complete amount of the Bacillus culture, and were given more feed after the complete consumption of the culture. The ingredient and nutrient composition of the basal diet was as follows. The forages of diet contained 30.5% chopped alfalfa hay and 9.5% maize silage. The concentrate consisted of 10.2% soybean meal, 16.8% cracked maize, 7.2% cottonseed meal, 12% corn distiller's grain solubles, 6% beer by-product, 1.8% molasses, 1.5% salt, 3% dicalcium phosphate, 0.6% mineral pre-mix (containing, per kg, 270 g calcium, 60 g magnesium, 40 g phosphorus, 40 g sodium, 5 g zinc, 4 g manganese, 1.5 g copper, 500 mg iodine, 50 mg cobalt, 15 mg selenium), 0.6% vitamin pre-mix (containing, per kg, 500 000 IU vitamin A, 100 000 IU vitamin D_3, and 500 IU vitamin E) and 1.3% limestone. The ratio of concentrate to forage was 40:60 (dry matter basis). The diet contained 6.80 MJ/kg net energy for lactation (NE_L, estimated by National Research Council, 2001), 166 g/kg crude protein (CP; AOAC, 1999), 304 g/kg acid detergent fibre (ADF) and 486 g/kg neutral detergent fibre (NDF), the latter two analysed according to Van Soest et al. (1991), with NDF being analysed without α-amylase and sodium

sulphite. Cows were fed twice daily. Treatment period lasted for 10 weeks. Individual feed intake and milk yield were recorded daily. Milk composition was analysed once weekly (am.-pm. composite) for percentage of protein, fat and lactose, using a Multispec Infrared Analyzer (Wheldrake, York, England). Cows were weighed at the start and at the end of the experiment and at monthly intervals until the study was completed.

Ruminal sampling

In experiment 2, three non-lactating Chinese Holstein cows with permanent fistulas and proximal duodenal cannula were used in the experiment. The experiment design was a 3 × 3 Latin square with 20-days periods, and test period was on the last 3 days at the end of each period. Cows were allowed twice daily *ad libitum* access to the same basal diet as cows in experiment 1, and were given free access to water. Cows in the treatment groups received 100 g Bacillus culture twice daily as a top-dress. Individual feed samples were collected prior to the a.m. (morning) and p.m. (evening) feeding for a 3 days period at the end of the experiment and composited to determine ruminal apparent nutrient digestibility. Samples were ground through a 1-mm screen and were analysed for dry matter (DM), crude protein (CP) (AOAC, 1999), ADF and NDF (as the feed samples).

The ruminal fluid samples were collected and analysed on the last 3 days of each period. Ruminal fluid was removed at 0.5 h, 1 h, 3 h and 6 h after morning feeding from the mid-ventral, anterior and dorsal regions. In addition, 750 ml of liquid from the ventral region was obtained and added to the grab samples for a total volume of approximately 4 l of ruminal contents. Ruminal contents were squeezed through four layers of cheesecloth and immediately analysed for pH by glass electrode. The pooled ruminal fluid sample was chilled to 5 °C with ice and transported to the laboratory. The ruminal fluid was centrifuged at 10 000 × *g* for 10 mins to remove feed particles and bacteria. The cell-free supernatant was stored at −20 °C. For analysis of ammonia nitrogen and volatile fatty acid (VFA), 1 ml of 25% metaphosphoric acid (wt/vol) was added to 5 ml of fermentation fluid and stored at −40 °C until analysed, and VFA analysed by gas chromatograph (model GC-2010, model 156 refractive index detector, model 421 CRT data controller, CR1A integrator, Bio-Rad HPX-87H organic acid column, loop, 0.013N H_2SO_4, 0.5 ml/min, 50 °C). For bacterial isolation, samples were thawed slowly at 4 °C and centrifuged (1000 × *g* for 10 mins at 4 °C) to remove protozoa and feed particles. The supernatant was collected and centrifuged (18 400 × *g* for 30 mins at 4 °C), washed with 25 ml of 0.9% saline and re-centrifuged (18 400 × *g* for 30 mins at 4 °C). The supernatant was discarded, and bacterial pellet was washed twice with 25 ml of deionized water and frozen at −20 °C. Bacterial isolates then were lyophilized and analysed for DM, ash, N and purines. The N:purine ratio was used to calculate the flow of bacterial N to the small intestine.

Duodenal sampling

In experiment 2, additionally duodenal digesta (approximately 500 ml) were collected every 4 h on day 18–20 of each period. Collection time advanced for 1 h each 24 h for a total of 18 samples. Stainless steel diversion tubes were inserted into the cannula during sampling to ensure that flow originated form the abomasums. Two hundred millilitre of digesta was added to a common container for each cow and frozen (−20 °C). Samples were thawed at room temperature (21 °C), and an aliquot was centrifuged (1000 × *g* for 10 mins at 4 °C). The remaining portion was lyophilized and ground through a 1-mm screen. Basal duodenal flow was determined using a dual marker system (Faichney, 1975), with Cr-EDTA (3.12 g/day) and $YbCl_3$ (1.12 g/day) as a liquid and solid marker, respectively, infused continuously into the rumen (from days 1 to 13). CP, organic matter (OM) (AOAC, 1999), ADF, NDF (Van Soest et al., 1991), Purine, N (Zinn and Owens, 1986) were determined in duodenal samples to calculate apparent digestibility in the rumen. The microbial duodenal flow was calculated using purine bases (PB):N of reconstituted digesta and rumen bacterial isolates [$(PB:N)_{chymus}/(PB:N)_{bacteria}$] (González-Ronquillo et al., 2004).

Statistical analyses

In experiment 1, data were analysed according to one-way ANOVA under a complete randomized design. Mean values were tested by Duncan's multiple range tests (Steel and Torrie, 1984). In experiment 2, data on various parameters were analysed using 3 × 3 Latin square design with general linear model procedure of SAS Institute (2003) The sums of squares were partitioned into animal, treatment and period. In both experiments, mean values were tested by Duncan's multiple range tests (Steel and Torrie, 1984). Significant differences were declared at $p < 0.05$.

Results

Milk production and composition

Both of the two Bacillus cultures did not alter feed intake ($p > 0.05$, Table 1) in Chinese Holstein cows. Mean daily yields of 4.0% fat corrected milk (FCM) and FCM/DM intake were significantly increased by *B. licheniformis* supplementation ($p < 0.05$, Table 1). Percentage of milk fat and lactose was not significantly different between treatments ($p > 0.05$). However, milk protein content increased with *B. licheniformis* supplementation ($p < 0.05$). In addition, mean body weight was unaffected by treatments ($p > 0.05$).

Ruminal apparent nutrient digestibility

The results of the ruminal apparent nutrient digestibility are shown in Table 2. Significant differences were found with OM, CP, NDF and ADF when cows were supplemented with *B. licheniformis* in comparison with the *B. subtilis* group and the control group ($p < 0.05$).

Rumen fermentation characteristics and microbial protein synthesis

Ruminal ammonia N concentration at all sampling time points after morning feeding was lower ($p < 0.05$, Table 3) in the cows receiving *B. licheniformis* supplementation. Ruminal ammonia N concentration at 1 h and 6 h was higher ($p < 0.05$) in cows receiving *B. subtilis* supplementation than in control group. There was a tendency that the cows fed *B. licheniformis* supplementation had a lower pH value ($p < 0.05$) at all sampling time points. *B. subtilis* supplementation had no effect on ruminal pH at all the sampling time points after feeding in comparison with the control group ($p > 0.05$). Total VFA and acetate concentrations were higher with *B. licheniformis* than in the other two groups ($p < 0.05$). *B. subtilis* supplementation had no significant effect on total and individual VFA compared to the control group ($p > 0.05$). *B. licheniformis* supplementation increased the non-ammonia nitrogen flow and duodenal microbial CP flow ($p < 0.05$) in experiment 2. *B. subtilis* supplementation did not increase the non-ammonia nitrogen flow and duodenal microbial CP flow ($p > 0.05$).

Table 1 Effect of Bacillus cultures on mean daily intake, milk yield, milk composition, feed conversion efficiency and body weight ($n = 10$)

	Treatments			
Item	Control	*Bacillus subtilis*	*Bacillus lichniformis*	SEM
Dry matter intake, kg/day	20.3	20.4	20.6	0.7
4.0% FCM*, kg/day	24.8[a]	24.2[a]	26.9[b]	1.6
Milk composition				
Fat, %	3.35	3.23	3.34	0.11
Protein, %	2.90[a]	2.93[a]	3.09[b]	0.05
Lactose, %	5.15	5.11	5.01	0.06
FCM/DM intake (kg/kg)	1.22[a]	1.19[a]	1.30[b]	0.6
Body weight, kg	568	575	579	19.6

*FCM, milk corrected to 4% fat.

a, b mean values within row bearing different superscripts differ significantly ($p < 0.05$).

Table 2 Effect of Bacillus culture supplementation on intake, duodenal passage, ruminal nutrient digestibility and microbial protein synthesis in Chinese Holstein cows ($n = 3$)

	Treatments			
Item	Control	*Bacillus subtilis*	*Bacillus lincheniformis*	SEM
Dry matter intake, kg/day	21.4	21.3	21.7	0.5
Organic matter				
Intake, kg/day	20.1	19.9	20.3	0.5
Passage to duodenum, kg/day	10.7	10.9	10.6	0.7
Apparently digested in the rumen, %	50.5[a]	49.7[a]	52.4[b]	0.3
Neutral detergent fibre				
Intake, kg/day	10.4	10.3	10.5	0.1
Passage to duodenum, kg/day	5.3	5.2	4.8	0.2
Apparently digested in the rumen, %	49.7[a]	49.5[a]	54.1[b]	1.0
Acid detergent fibre				
Intake, kg/day	6.5	6.4	6.6	0.1
Passage to duodenum, kg/day	3.4	3.4	3.2	0.2
Apparently digested in the rumen, %	47.7[a]	46.9[a]	51.5[b]	2.0
Nitrogen				
Intake, g/day	537	542	547	3.1
Passage to duodenum, g/day	428	437	467	4.3
Apparently digested in the rumen, %	20.2[a]	19.5[a]	14.6[b]	0.9
Bacterial N				
Passage to duodenum, g/day	224[a]	227[a]	259[b]	2.4
Bacteria N, % of N intake	41.7[a]	41.9[a]	47.3[b]	3.1

a, b mean values within row bearing different superscripts differ significantly ($p < 0.05$).

Discussion

Milk production and composition

Both the Bacillus cultures did not increase feed intake in Chinese Holstein cows, which indicated that feeding Bacillus cultures cannot increase the palatability of feeds. By contrast, Nocek et al. (2000,

Table 3 Ruminal fermentation characteristics of Chinese Holstein fed Bacillus cultures supplementation (n = 3)

	Treatments			
Parameters	Control	*Bacillus subtilis*	*Bacillus licheniformis*	SEM
Ammonia nitrogen (mg/dl)				
0.5 h	22.5[a]	21.6[a]	18.1[b]	0.63
1 h	22.8[a]	25.5[b]	18.6[c]	0.46
3 h	19.4[a]	20.4[a]	15.1[b]	0.68
6 h	12.6[a]	14.2[b]	11.2[c]	0.41
pH				
0.5 h	7.20[a]	7.10[a]	6.30[b]	0.14
1 h	6.82[a]	6.66[a]	6.40[b]	0.15
3 h	6.51[a]	6.50[a]	6.35[b]	0.12
6 h	6.91[a]	7.18[a]	6.42[b]	0.17
Total VFA* (mm/l)				
0.5 h	124[a]	130[a]	160[b]	1.9
1 h	125[a]	129[a]	159[b]	1.3
3 h	119[a]	121[a]	139[b]	0.9
6 h	115[a]	122[a]	137[b]	1.4
Acetate (mol 100 per mm)				
0.5 h	66.9[a]	67.3[a]	74.4[b]	0.91
1 h	53.3[a]	52.9[a]	64.1[b]	1.28
3 h	52.8[a]	50.7[a]	62.7[b]	0.77
6 h	50.7[a]	50.1[a]	58.3[b]	0.67
Propionate (mol 100 per mm)				
0.5 h	21.7	21.8	21.7	0.19
1 h	16.7	17.0	21.8	0.90
3 h	17.1	18.3	19.3	0.86
6 h	16.9	16.5	17.7	0.55
Butyrate (mol 100 per mm)				
0.5 h	10.0	9.9	10.2	0.99
1 h	7.8	9.1	9.5	0.72
3 h	7.8	7.8	8.1	0.64
6 h	7.8	7.9	7.9	0.11

*VFA, volatile fatty acid.

a, b, c mean values within row bearing different superscripts differ significantly ($p < 0.05$).

2003) as well as Soder and Holden (1999) reported that direct fed microbial increased dry matter intake in early lactation. Mean daily yields of FCM and FCM/DM intake were significantly affected by *B. licheniformis* supplementation. This result was similar with those of Wang et al. (2001) and Schingoethe et al. (2004). Although the percentage of milk fat was not significantly elevated by feeding *B. licheniformis*, *B. licheniformis* supplementation seems to stimulate fibre digesting microbes (see below). This slight discrepancy remains difficult to explain by the present study, but the phenomenon has been reported before (Piva et al., 1993). One possibility may be that the ADF concentration of the ration was sufficient to approach the genetical limit in milk fat synthesis of these cows in any case. Harri and Pekka (1996) reported that butyric acid also appeared to have a specific effect on milk fat content. Increased butyrate supply increased fat content, despite a constant ratio of acetate to propionate of approximately 3.5 and the ratio of (acetate + butyrate) to propionate >4.0 (Huhtanen et al., 1993), which is considered to be a threshold for milk fat content (Sutton, 1980). In experiment 2, ruminal butyrate concentration remained unchanged which therefore also might explain why the percentage of milk fat remained unchanged.

Milk protein was increased by *B. licheniformis* supplementation. A higher microbial protein synthesis in the rumen may result in a more efficient transfer of ruminal ammonia N into body and milk protein because of its high digestibility in the small intestine (Hvelplund and Hesselholt, 1987; Lapierre and Lobley, 2001; Blouin et al., 2002; Sarwar et al., 2004). This assumption was supported by the results of milk protein production in experiment 1. Some experiments showed that higher microbial crude protein in the intestine has a positive role in increasing the milk yield (Buttery and Foulds, 1985). As a result, microbial nitrogen that flowed into duodenum increased, and production of milk protein increased. *B. subtilis* supplementation increased ammonia nitrogen concentration at 6 h after morning feeding. This indicates that *B. subtilis* probably can stimulate protease producing microbes.

The present results indicated no effect of Bacillus cultures on milk lactose content. Hurtaud et al. (1993) and Miller-Webster et al. (2002) reported that lactose production in milk is associated with propionate production. Propionate is a major precursor of milk lactose. In our study, ruminal propionate concentration remained unchanged which therefore also might explain why the percentage of milk lactose remained unchanged.

Ruminal apparent nutrient digestibility

Some researchers (Nocek et al., 2002; Oellermann and Arambel, 1990; Wiedmtiel et al., 1987) reported that direct fed microbial culture supplementation can increase total tract digestibility of NDF and ADF. Ruminal digestibility of NDF and ADF in experiment 2 did support these results. Asshan and Qiao (2007) also reported that Bacillus cultures provide a source of vitamins, glucose, lactate, malate, formate, succinate and aspartate needed for bacterial growth and that, once lysis occurs, Bacillus protoplasm can be used as a source of nutrition for the rumen micro-organisms. In our study, *B. licheniformis* culture seemed to improve

fibre digestion in the rumen, which was confirmed in experiment 2. Krehbiel et al. (2003) reported that adding direct fed microbial culture in diets containing large amounts of concentrate increased the number and proportion of cellulose digesting bacteria in the rumen. This increase in cellulose digesting bacteria explains the improved ruminal digestibility of NDF and ADF.

Rumen fermentation characteristics and microbial protein synthesis

Mould and Ørskov (1993) and Wanapat et al. (2000) demonstrated that cellulose digestion is limited when ruminal pH reaches values below 6.0. Ruminal pH value was maintained between 6.30 and 7.20 across treatments in experiment 2. Ruminal VFA concentration is correlated with nutrient digestibility and pH (McDonald et al., 1995; Leng and Leonard, 1965) and this relation was also demonstrated in the present study (total VFA, nutrient digestibility and pH). Higher VFA and acetate concentrations using *B. licheniformis* supplementation were probably occurring because *B. licheniformis* stimulate cellulose digesting bacteria and more fibre was digested in the rumen. The same was reported by Ghorbani et al. (2002). However, supplementing the diet with *Bacillus cultures* did not increase the propionate in ruminal fluid compared with the control. Propionate production competes with methanogenesis for available hydrogen. Therefore, unchanged molar production of propionate across the treatment diets probably resulted in equal methane emission. This assumption was supported by the result of Asshan and Qiao (2007) who reported that Bacillus cultures had no effect on methane production *in vitro*. *B. subtilis* increased ammonia nitrogen concentration at 1 and 6 h which indicated that it has the ability to stimulate protein degrading bacteria in the rumen. The results of from this study suggest that bacillus culture may influence the individual VFA production in the rumen presumably by selective stimulation of the growth of certain species of anaerobic bacteria. Low ruminal pH may be caused by accumulation of VFA in the rumen. *B. licheniformis* decreased the ammonia nitrogen concentration at 0.5 h, 1 h, 3 h and 6 h after morning feeding. This indicated that probably more ammonia nitrogen was used to synthesis microbial nitrogen and may be the direct result of stimulated microbial activity. The ruminal ammonia reduction has usually been explained by increased microbial protein synthesis and enhanced ammonia assimilation (Khan et al., 2006; Bach et al., 2005; Alexander et al., 1996). Fraction and ratio of amino acids in bacteria are beneficial for ruminants (Storm and Ørskov, 1983), which can provide 35–65% metabolizable protein. Microbial protein synthesized in the rumen supplies the majority of absorbable amino acids in the small intestine, because rumen microbes form a large proportion of their cell protein from ammonia N (Hristov et al., 2004).

Conclusion

In conclusion, *B. licheniformis* used in this study was shown to be metabolically active and caused shifts in the microbial ecosystem of Chinese Holstein cows. The results of the study imply that the addition of *B. licheniformis* culture in the diet of Chinese Holstein cows is beneficial for performance. Further studies have to demonstrate these effects in other dairy breeds and diet compositions.

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