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Lactation performance and diet digestibility of dairy cows in response to the supplementation of *Bacillus subtilis* spores

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ABSTRACT

Bacillus subtilis is a transitory microorganism of the digestive tract, non-pathogenic to animals, and capable of forming spores that are resistant to heat and cold. As an animal feed probiotic the microorganism is supposedly capable of increasing diet digestibility and immunity. Two experiments were conducted to evaluate the effect of *Bacillus subtilis* spores on milk yield and composition and diet digestibility. In both experiments lactating dairy cows were fed in tie stalls and treatments were force-fed once per day. In experiment 1, 18 Holsteins in late lactation (246 ± 75 days in milk) received a sequence of the treatments *Bacillus subtilis* strain C-3102 (3.0×10^9 colony-forming units of spores per day) or Placebo in a crossover design with 39-day periods, a 10-day wash-out between periods, and response evaluated after the 28th day of the periods. The supplementation of *Bacillus subtilis* spores did not elicit detectable changes in intake (18.3 kg/d, $P = 0.91$), milk (25.3 kg/d, $P = 0.66$) and solids yield and concentration, total tract nutrient digestibility, and chewing activity. In experiment 2, 30 cows (161 ± 72 days in milk) with high milk somatic cell count (725,000 cells/mL) received the same treatments for 16 weeks, in a covariate adjusted randomized block design with repeated measures over time. *Bacillus subtilis* spores increased the yields of milk (25.3 *vs.* 23.6 kg/d, $P = 0.02$), protein (0.816 *vs.* 0.763 kg/d, $P = 0.01$), total solids (2.718 *vs.* 2.566 kg/d, $P = 0.05$), and energy (60.7 *versus* 56.5 MJ/d, $P = 0.02$) and milk urea-N tended to be reduced (19.3 *vs.* 20.8 mg/dL, $P = 0.06$). Milk somatic cell count did not differ between treatments. The positive lactation response to *Bacillus subtilis* spores supplementation occurred when the probiotic was fed for 16 weeks and there was no evidence to suggest that increased diet digestibility was a mediator of the response.

1. Introduction

Human food production from animals supplemented with live microorganisms as a replacement to chemical feed additives is in line with the natural trend of the consumer market. The addition of probiotics to the diet may have beneficial effect on gut physiology and immune function (Reid, 2008). Spore forming bacteria, such as *Bacillus subtilis*, have been used as probiotic supplements for humans and animals (Cutting, 2011). *Bacillus subtilis* is a transitory microorganism of the digestive tract, non-pathogenic to animals, and capable of forming spores resistant to heat and cold, having high stability in the diet (Sanders et al., 2003; Carlin, 2011; Cutting, 2011). The spores germinate at the intestinal lumen, which is required for its action as an animal feed probiotic. The bacterial population of *Bacillus subtilis* in the digestive tract is reduced after the cessation of the supplementation (Sanders et al., 2003). For this reason, the dietary supplementation of *Bacillus subtilis* should be performed daily. Probiotics based on bacterial spores can survive passage through the stomach (Hoa et al., 2000) with the potential to be more resistant to the low gastric pH compared with probiotic supplementation in the form of vegetative cells.

The supplementation of *Bacillus subtilis* has improved the performance of non-ruminants (Fritts et al., 2000; Hooge, 2008; Zhang et al., 2012, 2013; Lee et al., 2014) and calves (Sun et al., 2010). *Bacillus subtilis* can increase anaerobiosis in the digestive tract, which favors native proliferation of Lactobacilli capable of producing lactic acid and inhibiting pathogenic bacteria growth (Maruta et al., 1996; Sanders et al., 2003) and can improve immune function (Sun et al., 2010). Although increased diet digestibility is frequently proposed as a plausible response to spore forming bacterial supplements in non-ruminants, currently no study in lactating dairy cows have demon-

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strated this mechanism of action.

This research addresses the hypothesis that lactating dairy cows fed *Bacillus subtilis* can improve the lactational performance with positive changes in milk composition. Thus, two independent experiments were performed to investigate the response of dairy cows in lactation performance, milk composition, and diet digestibility to the supplementation with *Bacillus subtilis* spores.

2. Material and methods

The experimental procedures were in agreement with the ethical principles in animal experimentation of the Committee of Ethics in Animal Experimentation of the University of Paraná, Brazil (protocol number 050/2010).

2.1. Experiment 1

2.1.1. Cows, design, and treatments

Eighteen Holstein cows, with 246 ± 75 days in milk (DIM), 29.7 ± 5.8 kg/d of milk yield, and with somatic cell count (SCC) below 100,000 cells/mL at the start of the experiment, were individually fed in sand bedded tie stalls at the Better Nature Research Center (www.holandesflamma.com.br) and were millked three times per day in a adjacent herringbone parlor. Cows were paired blocked based on parity (primiparous and multiparous) and milk yield and were assigned to a sequence of two treatments in a crossover design, with 39-day periods. Between the two experimental periods, a 10-day washout was adopted, in which the animals received the same basal diet with no treatment addition. Response variables were evaluated on the last 12 days of each period.

Cows were fed on a Total Mixed Ration (TMR) mixed and offered twice daily starting at 0500 and 1300 h. The TMR was offered in amount sufficient to provide at least 10% of the offered as daily refusal. The composition of the TMR is reported in Table 1. Diet offered and orts from each cow were measured daily during the experiment. Treatments were 0.3 g/d of viable spores of *Bacillus subtilis* on calcium carbonate or Placebo (calcium carbonate). The active agent in the probiotic consisted of viable endospores of a single strain of *Bacillus subtilis* originally isolated from soil in Japan and dried and pasteurized to kill vegetative cells. The final product supplied *Bacillus subtilis* strain C-3102 (Calpis Co. Ltd., Tokyo, Japan) to provide a minimum daily intake of 3.0×10^9 cfu of viable spores. Gelatin capsules were filled with the treatments for daily oral dosing of each cow on return to their tie stalls after the morning milking.

Table 1
Composition of the offered total mixed ration in ingredients and of the consumed in nutrients (g/kg of dry matter) on treatments *Bacillus subtilis* spores or Placebo in Experiment 1 and of the basal diet in Experiment 2.

	Experiment 1		Experiment 2
	Bacillus subtilis	Placebo	
Corn silage	500		424
Tifton hay	41		
Soybean meal	202		218
Citrus pulp	102		158
High moisture corn	112		
Finely ground mature corn			156
Calcium salt of fatty acids	13		13
Urea	4		4
Magnesium oxide	3		
Limestone	8		8
NaCl	3		4
Sodium bicarbonate	8		9
Minerals and vitamins[a]	4		6
Crude protein	172	171	177
Neutral detergent fiber (aNDFom)	337	335	301
aNDFom from forage	268	267	221
Acid detergent fiber	238	238	215
Ether extract	48	48	52
Ash	93	93	88
Non-fiber carbohydrates[b]	350	353	385

[a] Composition per kg: 185 g of Ca; 150 g of P; 30 g of Mg; 30 g of S; 240 mg of Co; 3000 mg of Cu; 8000 mg of Mn; 12,000 mg of Zn; 90 mg of Se; 180 mg of I; 1000 KIU Vitamin A; 250 KIU Vitamin D3; 6250 IU Vitamin E.

[b] NFC (g/kg of dry matter) = 1000 − (crude protein + aNDFom + ether extract + ash).

2.1.2. Sample collection and analysis

Between days 28 and 39, samples of feed ingredients and orts from each cow were sampled daily and frozen. Composite samples per period were formed on an as fed basis for further analysis. The contents of dry matter (DM; method 934.01), organic matter (OM; ash method 924.05), crude protein (CP; method 984.13) and ether extract (EE; method 920.39) were analyzed according to the Association of Official Analytical Chemists (AOAC, 1990). Ash free neutral detergent fiber (aNDFom) was analyzed with porous crucibles following the recommendations of Van Soest et al. (1991) with heat-stable amylase and sodium sulfite. Acid detergent fiber was analyzed non-sequentially.

Samples from nine consecutive milkings were taken on days 29, 30, and 31 and a daily composite was formed in proportion to the volume secreted on each milking. The concentrations of protein, fat, lactose, and total solids were measured by infrared spectrophotometry using a Bentley 2000 analyzer (Bentley Instruments, Chaska, USA). The SCC was determined in a Somacount 150 equipment (Bentley Instruments, Chaska, USA) based on the principle of laser-based flow cytometry. Milk urea-N (MUN) was analyzed with a ChemSpec 150 (Bentley Instruments, Chaska, USA) that utilizes a modified Berthelot reaction. The daily secretion of milk energy (MJ/day) was calculated (NRC, 2001): {[(0.0929 × % fat) + (0.0547 × % protein) + (0.0395 × % lactose)] × kg of milk} × 4.184. The SCC was transformed to a linear scale from 0 to 9 (SCC score) in which scores represented the following values of SCC (×1000 cells/mL): 12.5 for SCC score 0; 25 for SCC score 1; 50 for SCC score 2; 100 for SCC score 3; 200 for SCC score 4; 400 for SCC score 5; 800 for SCC score 6; 1600 for SCC score 7; 3200 for SCC score 8; and 6400 for SCC score 9. The SCC score was calculated from the natural logarithm of the measured SCC (1000 cells/mL) and the above mentioned SCC value of each score: SCC score = −3.6438 + 1.4427 × Ln(SCC). Negative values were rounded to zero. Body weight (BW) and body condition score (BCS) were determined on day 35 to describe experimental units. The BCS was scored using a 1–5 scale, thin to fat (Wildman et al., 1982). The same three independent evaluators scored each cow. Cows were weighed immediately after the morning milking.

The total tract apparent digestibility coefficient of DM, OM, aNDF, and Non-aNDF OM was determined on days 33–35 by total fecal collection. Feces were collected concurrent to defecation during three 8-h sampling periods and weighed. The second and third sampling periods were each delayed by 8 h to avoid a major disturbance to the animals, while still representing a 24-h collection period. Fecal aliquots (equal fresh weight basis) were immediately frozen along the collection period and a composite sample was formed. Daily digestible OM intake (DOMI, kg/day) was calculated. Feed efficiency was determined as the ratio of milk yield to DM intake. The efficiency of energy use was defined by the ratio between milk energy (MJ/day) and DOMI.

On day 28, chewing activity was assessed by visual observation of the buccal activity of each cow, at 5-min intervals, continuously for 24 h. Chewing time (min/ day) was defined as the sum of ingestion and rumination times. Chewing, ingestion, and rumination per unit of DM intake were calculated using the DM intake of the day in which chewing activity was evaluated.