Effects of lactic acid bacteria silage inoculation on methane emission and productivity of Holstein Friesian dairy cattle

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ABSTRACT

Inoculants of lactic acid bacteria (LAB) are used to improve silage quality and prevent spoilage via increased production of lactic acid and other organic acids and a rapid decline in silage pH. The addition of LAB inoculants to silage has been associated with increases in silage digestibility, dry matter intake (DMI), and milk yield. Given the potential change in silage and rumen fermentation conditions accompanying these silage additives, the aim of this study was to investigate the effect of LAB silage inoculants on DMI, digestibility, milk yield, milk composition, and methane (CH₄) production from dairy cows in vivo. Eight mid-lactation Holstein-Friesian dairy cows were grouped into 2 blocks of 4 cows (multiparous and primiparous) and used in a 4 × 4 double Latin square design with 21-d periods. Methane emissions were measured by indirect calorimetry. Treatments were grass silage (mainly ryegrass) with no inoculant (GS), with a long-term inoculant (applied at harvest; GS+L), with a short-term inoculant (applied 16 h before feeding; GS+S), or with both long and short-term inoculants (GS+L+S). All diets consisted of grass silage and concentrate (75:25 on a dry matter basis). The long-term inoculant consisted of a 10:20:70 mixture of *Lactobacillus plantarum*, *Lactococcus lactis*, and *Lactobacillus buchneri*, and the short-term inoculant was a preparation of *Lc. lactis*. Dry matter intake was not affected by long-term or short-term silage inoculation, nor was dietary neutral detergent fiber or fat digestibility, or N or energy balance. Milk composition (except milk urea) and fat and protein-corrected milk yield were not affected by long- or short-term silage inoculation, nor was milk microbial count. However, milk yield tended to be greater with long-term silage inoculation. Methane expressed in units of grams per day, grams per kilogram of DMI,

grams per kilogram of milk, or grams per kilogram of fat and protein-corrected milk yield was not affected by long- or short-term silage inoculation. However, CH₄ expressed in units of kilojoules per kilogram of metabolic body weight per day tended to be greater with long-term silage inoculation. Results of this study indicate minimal responses in animal performance to both long- and short-term inoculation of grass silage with LAB. Strain and dose differences as well as different basal silages and ensiling conditions are likely responsible for the lack of significant effects observed here, although positive effects have been observed in other studies.

Key words: lactating cows, lactic acid bacteria, silage inoculant, methane

INTRODUCTION

Microbial silage inoculants such as lactic acid bacteria (LAB) are used to improve silage fermentation and prevent spoilage of ryegrass and maize silages through increased organic acid production, mainly lactic acid (LA) and acetic acid, and a more rapid pH decline (Muck, 2013). Although plants contain native LAB, the number of viable LAB on forage can be insufficient and delay the decline in pH during ensiling, allowing greater loss of nutrients, other microbes to dominate fermentation, or both. Common LAB used for silage inoculation include homofermentative species such as *Lactobacillus plantarum*, *Enterococcus faecium*, and *Pediococcus* spp. Ensiling with these LAB results in conversion of water-soluble carbohydrate (WSC) to LA and a rapid decline in silage pH under anaerobic conditions (McDonald et al., 1991), which helps to prevent the development of clostridia, yeasts, molds, and fungi (Muck, 2013). Nutritionally, because of a more rapid pH decline, LAB inoculants tend to reduce the ammonia N content of silage via reduced fermentation of AA and reduced protein breakdown, as well as improved DM recovery (McDonald et al., 1991; Spoelstra, 1991; Henderson, 1993). Other heterofermentative LAB

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(e.g., *Lactobacillus buchneri*) that grow more slowly and produce a greater concentration of acetic acid are also often included and are typically inhibitory to fungi and preserve silage susceptible to spoilage upon exposure to air (e.g., Driehuis et al., 1999).

The use of LAB silage inoculants has been associated with increases in DMI, digestibility, and milk yield, although the mechanism remains convoluted (Weinberg and Muck, 1996; Weinberg et al., 2003; Ando et al., 2006). In addition, LAB have also been postulated as a potential probiotic with mastitis mitigation potential (e.g., Fang et al., 1996). Across all effects, results appear to be strain, dose, and silage specific. For example, an early meta-analysis (Muck and Kung, 1997) demonstrated positive responses in DMI to microbial inoculants 28% of the time, in body weight gain 53% of the time, and in milk production 47% of the time, based on literature from 1990 to 1995. Several hypotheses exist on the cause of improved animal performance, when observed, including (1) changes in the chemical composition of the silage, (2) inhibition of detrimental microbes and the production of toxins (e.g., bacteriocins; Gollop et al., 2005), and (3) interaction of LAB with rumen microbes and alteration of rumen fermentation (Fellner et al., 2001; Weinberg et al., 2003). The hypothesis for mastitis mitigation generally revolves around reduction of milk pH and competition for nutrients with detrimental bacteria (e.g., Fang et al., 1996), but the pathway by which oral LAB probiotics may reach the mammary gland is uncertain and at times convoluted (Fernández et al., 2013).

Nutritional or microbial changes within the rumen may also affect enteric methane (CH_4) production. Methane is a greenhouse gas produced as the result of enteric fermentation in the ruminant digestive tract, representing an energetic loss of 2 to 12% (Ellis et al., 2008). Globally, enteric fermentation from domestic ruminants accounts for ~6.0% of the global anthropogenic CO_2 equivalent production (FAOSTAT, 2013). Improving forage (either fresh or conserved) quality and digestibility is among the most effective mitigation strategies to decrease CH_4 emission intensity (CH_4 per unit product; e.g., Hristov et al., 2013), which makes examining the potential impact of silage inoculants relevant. So far, several in vitro studies have suggested reductions in total gas production [Muck et al. (2007) using alfalfa silage and considering 14 individual and mixed LAB treatments] or CH_4 production [Cao et al. (2010), using whole-crop rice silage with *Lb. plantarum*; Cao et al. (2011), using vegetable residue silage with *Lb. plantarum*] without changes in digestibility. However, not all in vitro studies have reported reductions in CH_4 [Contreras-Govea et al. (2011), using alfalfa and corn silage, with individual and mixed LAB treat-

ments], likely pointing to strain and inoculant differences, substrate differences, or in vitro methodology differences. Indeed, Ellis et al. (2016) found that LAB silage inoculants in vitro were effective on grass silage and not corn silage, and that some strains increased but others decreased (or did not change) total gas production, CH_4 production, or DM digestibility.

Based on these in vitro results, the purpose of this study was to examine in vivo the long-term use (applied at harvesting, a 10:20:70 cfu/g product ratio of *Lb. plantarum*, *Lc. lactis*, and *Lb. buchneri*) and short-term use (applied 16 h before feeding, *Lc. lactis* as a probiotic) of LAB inoculants applied to grass silage for its effects on CH_4 emission, DMI, diet digestibility, milk yield, milk composition, and milk microbial changes. The long-term inoculant was applied to examine potential effects due to changes in silage fermentation and silage composition, and the short-term inoculant was applied to examine any direct probiotic effects. We hypothesized that LAB may have direct or indirect CH_4 mitigation potential in vivo when applied to grass silage fed to dairy cattle.

MATERIALS AND METHODS

This study was conducted at the Carus animal facility at Wageningen University, Wageningen, the Netherlands, in accordance with Dutch law and approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands).

Cows, Experimental Design, and Diets

Eight mid-lactation Holstein-Friesian dairy cows were grouped into 2 blocks of 4 cows and used in a 4×4 double Latin square design (21-d periods). Cows within blocks were matched for age, parity, stage of lactation, and milk production. The cows in block 1 (multiparous, average 4.5 ± 0.25 lactations) averaged 95 ± 18.0 DIM at the start of the experiment with an average BW of 676 ± 20.2 kg, and the cows in block 2 (primiparous) averaged 112 ± 2.0 DIM at the start of the experiment with an average BW of 521 ± 9.4 kg. Prior to the onset of the experiment, animals were group-housed for 7 d and fed a regular dairy ration while acclimatizing to the experimental location. Periods for blocks 1 and 2 were staggered by 4 d to facilitate measurements with the 4 individual climate respiration chambers available.

The experimental diet consisted of 75% grass silage (mainly *Lolium perenne*) and 25% concentrate mixture [concentrate, g/kg product basis: 242 maize, 64 wheat, 10 linseed, 84 palm kernel expeller, 34 formaldehyde-treated rapeseed meal, 72 rapeseed meal, 259 formaldehyde-treated soybean meal, 100 beet pulp, 14.4 lime, 2.8

salt, 50 molasses, 35 vinasse, 5.0 urea, 12.5 Mervit 2019 Cattle CPL (commercial premix; Nuscience, Utrecht, the Netherlands), 10 Rupromin balance (commercial premix; Provimi, Rotterdam, the Netherlands), 5.0 Mervit 2325 Sporavit (commercial premix; Nuscience)]. Treatments were based on feeding grass silage with or without a long-term and short-term LAB inoculation as follows: (1) control grass silage with no inoculation (**GS**), (2) grass silage inoculated with LAB at the time of grass harvesting (long-term inoculant; **GS+L**), (3) grass silage plus a LAB inoculant applied 16 h before feeding (short-term inoculant; **GS+S**), and (4) grass silage plus both the short-term and long-term LAB inoculants (**GS+L+S**).

The grass for the experimental silages was harvested from a 10-ha plot of diploid perennial ryegrass (*L. perenne*) cultivars of intermediate- and late-heading type (36% each) and timothy grass (*Phleum pratense*; 28%); for further details, see Warner et al. (2016). The grass mixture was a second-cut fall harvest with visible onset of crown rust (*Puccinia coronata*). Regrowth period was 6 wk. The grass was cut on the afternoon of September 4, 2012, left to wilt 1 d, and then inoculated and baled (chopped to 4 cm) using a Krone Big Pack 1270 XC (Krone, Spelle, Germany). Bales were wrapped in the field using stretch film (Vischer-Holland Megastretch stretch film, Steenwijk, the Netherlands; 750 mm wide and 25 μ m thick) in 16 turns of the bale with a 50% overlap. The grass DM at time of harvest was 45%. Control grass (no inoculation; only water applied) was baled first to avoid contamination with the LAB inoculant, and bales were collected from grass spaced across the entire plot. For distribution of the LAB inoculant into the treated bales, a tractor mounted 400-L tank was used, and the inoculant was added directly to the water immediately before baling commenced. The hose of the tank was connected to a boom containing 2 nozzles, the boom was mounted slightly above the pickup of the baler, and the nozzles were directed slightly into the baler. Based on calibration data, an application rate of 0.61 L of liquid was applied per metric tonne of fresh forage. The control grass received an equal amount of water applied to ensure identical handling. Both control and treated bales were then stored outside for a minimum of 7 mo, until the onset of the trial on April 1, 2013.

The long-term inoculant was prepared by Chr. Hansen (Hørsholm, Denmark) as a powdered mixture of 10:20:70 (cfu/g of product) *Lb. plantarum* (6072), *Lc. lactis* (O-224), and *Lb. buchneri* (LB1819), respectively. The inoculant was applied in a water solution during grass harvest to achieve the standard of 1.5×10^5 cfu/g of grass as applied in current practice. Generally, an

inoculation rate of 10^5 to 10^6 viable cells per gram of crop is sufficient for the inoculant LAB to overwhelm the epiphytic LAB and become the predominant population in the silage (Weinberg and Muck, 1996). The short-term inoculant, also prepared by Christian Hansen, was a powdered preparation of *Lc. lactis* (SR3.54). The short-term inoculant was applied to the grass silage in a water solution 16 h before morning feeding to obtain an intake of 5×10^9 cfu/cow per day. The short-term inoculant was only applied to the morning feed, which was restricted to 95% of ad libitum morning intake to ensure complete consumption of the LAB offered. The afternoon feed was ad libitum and hence intake remained ad libitum on a daily basis. Cows were housed in individual tie-stalls or individual respiration chambers during the full course of the experiment. Drinking water was provided ad libitum during the entire experiment.

Methane Measurement

In each 21-d period, 17 d of adaptation to the experimental diet was allowed, during which cows were individually housed in tie-stalls (Carus Research Facilities, Wageningen University, Wageningen, the Netherlands). From d 18 (1500 h) through d 22 (0900 h), animals were individually housed in 1 of 4 identical climate respiration chambers (**CRC**) at the Carus Research Facilities. A detailed description of the Wageningen University CRC system has recently been given by van Gastelen et al. (2015). Briefly, in each CRC (volume of 35 m³) relative humidity was maintained at 70% and temperature at 16°C, and cows were exposed to 16 h of light per day. The lighting regimens were identical between the tie-stall barn and the CRC, and a night light was on between 2230 and 0530 h. The ventilation rate was 42 m³/h per compartment, and inlet and exhaust air of each compartment was sampled at 10-min intervals. Gas concentrations and ventilation rates were corrected for pressure (101.3 kPa), temperature (273.15 K), and humidity (65%) to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Staff entered each CRC compartment twice daily at 0600 and 1600 h for approximately 30 min for milking and feeding. The gas measurements during the opening of the CRC were not used for data analysis. Cows were weighed immediately upon entering and just before leaving the CRC on a built-in scale in each chamber. Measurement of CH₄ production, diet digestibility, energy and N balance, feed intake, milk production, and milk composition as well as metabolites in blood and the microbial composition of milk were taken during the chamber period.

Sampling and Measurements

Feed Intake, Digestibility, and Balance Data.

Silage and concentrate samples were collected during the measurement period in the CRC and pooled by period. Orts were collected each morning and afternoon and stored at 4°C, pooled per cow during the measurement period, and stored at -20°C pending analyses. Excreta (feces plus urine) were quantitatively collected from the CRC per cow after the measurement period and homogenized, and subsamples of ~500 mL were stored at -20°C. Respiration gases and condensed water from the CRC were collected and stored at 4°C pending analyses. While cows were in the chambers, total collection of feed refusals, milk, and manure was conducted so that N and energy balance could be determined. Assuming that urine does not contain significant amounts of NDF and fat, this collection also allowed determination of NDF and fat digestibility.

Milk Production and Milk Composition. Cows were milked twice daily at 0600 and 1600 h in the CRC, and milk production was recorded at each milking. Milk was collected twice daily during milkings. A milk sample (10 mL) of each milking was collected in a tube containing sodium azide (5 µL) for preservation and analysis for fat, protein, and lactose content and cell count, and milk samples for fatty acid (FA) analysis were collected with the same procedure but pooled per cow per period. Milk samples were also taken at each milking (5 g/kg of milk), pooled per cow per period, and stored at -20°C pending milk energy, MUN, and N analyses. Pooled samples were prepared with correction for differences in milk yield between individual milking events.

Blood Metabolite Variables. Blood samples, extracted from the coccygeal blood vessel, were taken before morning feeding/milking (0600 h) on d 17 of the experiment (the day before cows entered the respiration chambers) for analysis of glucose, insulin, BHB, and nonesterified fatty acid (NEFA) content in blood.

Milk Microbial Composition. Milk samples were collected on d 4 of the chamber period into sterile tubes at the 0600 and 1600 h milking to determine the microbial population present. The PathoProof Mastitis PCR assay (Thermo Scientific, Roskilde, Denmark) was performed directly on milk samples to detect the presence of *Klebsiella* spp., *Serratia marcescens*, *Arcanobacterium pyogenes* and *Peptoniphilus indolicus*, and yeast. A cycle threshold (Ct) value of 40 indicates that the microbe was not detectable. Values of 36 to 39 correspond to a quantity of approximately 10 pathogens/mL of milk (1 log/mL of milk), and values of 32 to 35 correspond to approximately 100 pathogens/mL of milk (2 log/mL of milk).

Microbial Counts in Silage

Total counts of yeast and mold (cfu/g) were determined by spread plating diluted samples on YGC agar plates and counting colonies after a 5-d incubation at 25°C and 37°C for yeast and mold, respectively.

Chemical Analysis

All feed, Orts, total excreta, and feces samples were oven-dried at 60°C and ground to pass a 1-mm sieve (Peppink 100 AN, Peppink, Olst, the Netherlands) before analyses. Subsamples were analyzed by wet chemistry according to the methods described in detail by Abrahamse et al. (2008). Grass and concentrate samples were analyzed in duplicate for DM, ash, N, crude fat, starch (concentrate only), reducing sugars (all carbohydrates with reducing properties and soluble in 40% ethanol), NDF, ADF, ADL, and gross energy (GE). Orts were analyzed for DM and ash. Total excreta samples were analyzed for DM, ash, fat, NDF, and GE. Dry matter content of samples were determined by drying at 103°C (ISO 6496; ISO-IDF, 1999b) and ash content by combustion at 550°C (ISO 5984; ISO-IDF, 2002a). Sample GE content was determined by bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany) according to ISO 9831 (ISO-IDF, 1998). The Berntrop-method (ISO 6492; ISO-IDF, 1999a) was used to determine crude fat, and N was determined according to the Kjeldahl method with CuSO₄ as the catalyst (ISO 5983-2; ISO-IDF, 2009). The NDF content of samples were determined according to a modification of Van Soest et al. (1991), with additional incubations in α-amylase and protease as described by Goelema et al. (1998). Reducing sugar analysis was carried out as described by Van Vuuren et al. (1993) using a 40% ethanol solution. Modifications to this method were as follows: (1) after hydrolysis, 0.25 M K₄Fe(CN)₆·3H₂O (Carrez I) and 0.5 M Zn(C₂H₃O₂)₂·22H₂O (Carrez II) were used to clarify the solution, which was a filtered (595¹/₂, 150-mm diameter 2) dilution dependent on estimated concentrations of sugars, which were dissolved in 10-fold lower concentrations of CuSO₄·5H₂O, Na₂CO₃·10H₂O, and neocuproine; (2) concentrations were measured using a spectrophotometer (model Du 530, Beckman Coulter, Fullerton, CA) at 460 nm; and (3) concentrations were estimated by calibration using a standard curve ranging from 10 to 100 mg of glucose/L. Starch content of samples were determined enzymatically with dimethyl sulfoxide solubilization according to ISO 15914 (ISO-IDF, 2004b).

Silage and milk samples were further analyzed for FA composition. For FA analysis in silage, samples were extracted with chloroform/methanol (2:1, vol/

vol) containing tridecanoic acid (C13:0) as the internal standard, as described by Khan et al. (2009). Fatty acids were methylated by a 2-step procedure. The first methylation step (basic) was performed by adding 3 mL 0.5 *N* NaOH and heating for 30 min at 50°C. For the second methylation step (acid), 2 mL HCl/methanol (1:1, vol/vol) was added and heated for 10 min at 50°C. After cooling, 2 mL of hexane and 2 mL of distilled water were added to the mixtures, which were then mixed and centrifuged at 800 *g* for 5 min at 20°C. The hexane layer was removed, and another 2 mL of hexane was added to the aqueous layer, which was mixed and then centrifuged at 800 $\times$ *g* for 5 min at 20°C. Both hexane fractions were combined and evaporated to dryness under an N₂ flux and resolve in 1 mL of hexane. Methyl fatty acids were separated and detected by GC-flame ionization detector and quantified by the internal standard procedure as described by Khan et al. (2009). Milk fat was extracted from the milk samples and fatty acid methyl esters were prepared from fat fractions (ISO 15884; ISO-IDF, 2002b). Methyl esters were analyzed (ISO 15885; ISO-IDF, 2002c) on a TRACE Ultra gas chromatograph (Thermo Electron Corporation, Waltham, MA) with a split/splitless injector operated in split mode (split ratio 1:100), at a temperature of 270°C, using a Varian WCOT fused silica column with CP-select 258 CB for fatty acid methyl esters as stationary phase (100 m $\times$ 0.25 mm i.d.; Varian Inc., Palo Alto, CA) and hydrogen as carrier gas, and fitted to a flame ionization detector (250°C). The initial temperature was held at 65°C for 1 min, increased to 225°C at 3°C/min, and held at 225°C for 5 min. A volume of 1 μ L was injected. Peaks were identified and quantified using pure methyl esters (Sigma-Aldrich, Zwijndrecht, the Netherlands; Larodan, Malmo, Sweden; Lipidox).

Milk composition was analyzed by mid-infrared reflection spectroscopy at VVB Milk Control Station (Nunspeet, the Netherlands) using standard procedures (ISO 9622; ISO-IDF, 1999c) for GE and N content as described previously and for urea content based on the enzymatic method using difference in pH (ISO 14637; ISO-IDF, 2004).

From the CRC unit, samples of condensed water (i.e., collected from the heat exchanger) and air (i.e., through which the outflowing air was led, to trap aerial ammonia) were analyzed for N, in a 25% sulfuric acid solution (wt/wt) of each.

Statistical Analysis

For statistical analysis, treatments were blocked as long term (yes/no) and short term (yes/no), so that long-term, short-term, and interaction effects could be analyzed. Data were analyzed using PROC MIXED

of SAS (SAS 9.3; SAS Institute, 2011). The statistical model included the fixed effects of treatment (divided into long term and short term), period, and block, and cow (block) was treated as a random effect. Nonsignificant interaction terms were removed from the model. The fixed effect of chamber was not significant and thus not included. All results reported are least squares means, and significant effects were declared when $P \leq 0.05$, and tendencies when $0.05 \leq P \leq 0.10$.

RESULTS

Silage and Diet Composition

Chemical composition of the experimental silages and grain are summarized in Tables 1 and 2. The treatment silage was slightly lower in CP and reducing sugars, and similar in NDF and ADF, compared to the control silage (Table 1). The silage pH, LA, acetic acid, and ammonia content of control and treated silages were similar (Table 1). The FA composition of silages, as reported in Table 2, was largely similar between control and treated silages.

Chemical composition of the experimental diets is summarized in Table 3. Diets with long-term inoculants (GS+L and GS+S+L) were slightly lower in CP and reducing sugars and slightly greater in starch compared to the control diets (GS and GS+S), but short-term inoculated diets (GS+S and GS+S+L) did not differ greatly from the control diets (GS and GS+L).

Intake, Milk Yield, Milk Composition, and Methane Emissions

Dry matter intake and BW were not affected by long-term or short-term silage inoculation (Table 4). Milk yield (kg/d) and fat- and protein-corrected milk yield (FPCM; kg/d) were not affected by short-term silage inoculation in this study. However, milk yield tended ($P = 0.083$) to be greater with long-term silage inoculation. Similarly, milk CP, lactose, and fat (kg/d and %) did not significantly differ from control values with long- or short-term inoculations. However, a trend was seen for greater milk lactose yield (kg/d) ($P = 0.066$) with long-term inoculation that coincided with the trend for greater milk yield.

Milk urea was significantly lower with the long-term inoculation ($P = 0.027$; Table 4), coinciding with lower dietary CP content (Table 3). The efficiency of milk N production (N milk, % N intake) was not affected (Table 5).

Several of the long-chain FA in milk differed with long- and short-term inoculations (Table 6). A trend existed for the UFA content of milk to be lower with

Table 1. Chemical composition (g/kg of DM unless otherwise noted) of grass silages and concentrate

Item	Experimental grass silage		Concentrate
	Control	Treated	
Chemical composition			
DM, g/kg	463	461	910
OM	900	901	902
CP	174	167	245
NDF	476	478	158
ADF	283	288	103
ADL	21	20	26
Reducing sugars	61	55	100
Starch	—	—	231
Fat	44	42	25
Gross energy, MJ/kg of DM	18.9	18.8	17.5
Ensiling characteristics			
pH	4.7	4.8	—
Lactic acid	45	40	—
Acetic acid	13	13	—
Ammonia	2.8	2.7	—

long-term silage inoculation ($P = 0.080$), and the PUFA content was significantly lower ($P = 0.007$). The C18:1 *trans*-11 FA content in milk was lower ($P = 0.008$) with long-term inoculation, suggesting that the last step of biohydrogenation (C18:1 *trans*-11 to C18:0) was more efficient in the digestive tract. Similarly, C18:3 *cis*-9,12,15 and C18:0 were also lower with the long-term inoculation. These changes occurred despite minimal differences in silage FA composition (Table 2), suggesting that differences are due to changes in rumen digestion and fermentation.

Methane emission expressed in units of grams per day, grams per kilogram of DMI, grams per kilogram of milk yield, or grams per kilogram of FPCM was not af-

fected by long- or short-term silage inoculation (Table 4).

Diet Digestibility, N Balance, and Energy Balance

Dietary NDF and fat digestibility was not affected by long-term or short-term LAB inoculation (Table 5). Similarly, treatments had no effects on N balance or energy balance. However, within the energy balance analysis, a trend ($P = 0.062$) existed for CH₄ production (kJ/kg of BW^{0.75} per day) to be greater with the long-term inoculation as well as for heat production (kJ/kg of BW^{0.75} per day), coinciding with the numerically greater GE intake (kJ/kg of BW^{0.75} per day).

Blood Metabolites

The blood metabolites glucose, NEFA, BHB, and insulin were not affected by long- or short-term silage inoculation (Table 7).

Milk Microbial Analysis

Results of Ct analysis, indicating the presence of microorganisms in milk, are presented in Table 8. *Klebsiella* spp. analysis had only 2 values different from 40 and yeast Ct data had no deviations from 40, indicating undetectable quantities; therefore, no statistical analysis was warranted for these microbes. For *Serratia marcescens* and for *Arcanobacterium pyogenes* and *Peptoniphilus indolicus* or both, neither long-term nor short-term treatment had a significant effect on Ct values obtained for these microbes (Table 8). All treatment means had Ct values greater than 35, indicating low presence in milk. Total pathogen load (the sum of

Table 2. Long-chain fatty acid (FA) composition of control and inoculant-treated grass silages

Silage fatty acid, g/kg of DM	Grass silage	
	Control	Treated
Individual FA		
C14:0	0.12	0.13
C14:0 <i>anteiso</i>	0.59	0.55
C16:0	3.83	3.52
C16:0 <i>anteiso</i>	0.75	0.72
C16:1	0.10	0.06
C18:0	0.38	0.30
C18:1 <i>cis</i> -9	0.60	0.52
C18:1 <i>cis</i> -11	0.10	0.08
C18:2 <i>cis</i> -9,12	3.12	3.06
C18:2 <i>trans</i> -10, <i>cis</i> -12	0.10	0.07
C18:3 <i>cis</i> -9,12,15	8.48	8.76
Summary		
Total FA	18.90	18.32
SFA	6.24	5.67
UFA	12.66	12.65
MUFA	0.80	0.65
PUFA	11.86	11.99

Table 3. Chemical composition, gross energy value, and forage-to-concentrate ratio of treatment diets

Variable	Treatment diet ¹			
	GS	GS+S	GS+L	GS+S+L
Chemical composition, g/kg of DM				
OM	900	900	901	901
CP	192	192	187	187
NDF	396	398	395	394
ADF	238	239	240	239
ADL	22	22	22	22
Reducing sugars	71	71	66	67
Starch	58	57	60	61
Fat	39	39	38	38
Gross energy, MJ/kg of DM	18.5	18.5	18.4	18.4
Forage:concentrate	0.75	0.76	0.74	0.74

¹GS = grass silage (mainly ryegrass) with no inoculant; GS+S = grass silage with a short-term inoculant (applied 16 h before feeding); GS+L = grass silage with a long-term inoculant (applied at harvest); GS+S+L = grass silage with both long- and short-term inoculants.

Ct values for *Klebsiella* spp., *Serratia marcescens*, and *Arcanobacterium pyogenes* and *Peptoniphilus indolicus* or both and yeast) was also not significantly affected by treatment (Table 8). Milk SCC did not differ with long- or short-term silage inoculation either (Table 4) but was numerically lower with the short-term inoculation.

DISCUSSION

DMI, Diet Digestibility, and Milk Yield

In the current study, no significant effect of LAB inoculation was observed on DMI. Dry matter intake

responses reported in the literature to LAB silage inoculations are variable. Some studies show increases in DMI with inoculated corn (Kung et al., 1993; Basso et al., 2014) and grass silages (Gordon, 1989; Mayne, 1990; Petit and Flipot, 1990; Meeske et al., 1999), but others have shown no effect with corn silage (Wardynski et al., 1993; Fellner et al., 2001), grass silage (Keady et al., 1994; Keady and Murphy, 1996), or barley silage (McAllister et al., 1995). In the studies in which positive responses in DMI were observed, they were often attributed to improved NDF digestibility or more favorable fermentation conditions such as pH, but not always (Bayatkouhsar et al., 2011). In agreement with

Table 4. Dry matter intake, BW, milk yield, milk composition, and methane emissions from cows fed diets with or without long-term and short-term silage inoculation with lactic acid bacteria

Variable	Treatment diet ¹				SEM	P-value		
	GS	GS+S	GS+L	GS+S+L		Long-term	Short-term	Long-term × short-term
DMI, kg/d	15.9	16.4	16.4	16.4	0.88	0.402	0.401	0.495
BW, kg	584	586	590	585	13.8	0.403	0.707	0.322
Milk yield, kg/d	22.1	22.2	22.6	22.9	0.77	0.083	0.544	0.845
FPCM, ² kg/d	22.5	22.7	23.3	23.2	0.76	0.143	0.908	0.749
Milk CP, kg/d	0.67	0.69	0.70	0.70	0.029	0.135	0.390	0.687
Milk CP, %	3.10	3.14	3.12	3.11	0.069	0.857	0.431	0.268
Milk lactose, kg/d	1.00	1.00	1.02	1.04	0.033	0.066	0.644	0.487
Milk lactose, %	4.55	4.51	4.53	4.57	0.04	0.213	0.916	0.024
Milk fat, kg/d	0.95	0.96	0.99	0.97	0.036	0.389	0.793	0.714
Milk fat, %	4.31	4.31	4.33	4.27	0.137	0.932	0.855	0.825
Milk urea, mg/dL	29.6	29.4	27.0	26.2	2.53	0.027	0.625	0.801
Milk SCC ³	139	126	158	104	70.7	0.954	0.280	0.508
CH ₄ , g/d	352	353	359	366	18.3	0.120	0.526	0.599
CH ₄ , g/kg of DMI	22.1	21.6	22.0	22.3	0.51	0.109	0.618	0.045
CH ₄ , g/kg of milk	16.3	16.1	16.2	16.2	0.66	0.839	0.523	0.532
CH ₄ , g/kg of FPCM	16.0	15.6	15.8	15.8	0.62	0.993	0.641	0.595

¹GS = grass silage (mainly ryegrass) with no inoculant; GS+S = grass silage with a short-term inoculant (applied 16 h before feeding); GS+L = grass silage with a long-term inoculant (applied at harvest); GS+S+L = grass silage with both long- and short-term inoculants.

²FPCM = fat- and protein-corrected milk yield.

³Cells × 1,000/mL.

Table 5. Digestibility, N, and energy balance from cows fed diets with or without long-term and short-term silage inoculation with lactic acid bacteria

Variable	Treatment diet ¹				SEM	P-value		
	GS	GS+S	GS+L	GS+S+L		Long-term	Short-term	Long-term × short-term
NDF digestibility %	70.0	71.4	71.7	70.8	1.30	0.673	0.846	0.352
Fat digestibility %	39.5	42.4	40.4	38.7	1.95	0.483	0.742	0.244
N balance								
N intake, mg/kg of BW ^{0.75} per day	4,129	4,235	4,155	4,085	212	0.829	0.277	0.525
N manure, mg/kg of BW ^{0.75} per day	2,780	2,778	2,744	2,759	137	0.579	0.887	0.867
N manure, % N intake	67.1	65.2	66.2	67.3	1.07	0.548	0.682	0.147
N milk, mg/kg of BW ^{0.75} per day	883	909	944	870	45.0	0.725	0.183	0.048
N milk, % of N intake	21.4	21.7	22.4	21.6	0.87	0.453	0.757	0.504
N retained, ² mg/kg of BW ^{0.75} per day	399	496	433	414	68.5	0.670	0.497	0.323
N retained, % of N intake	9.5	11.8	10.1	10.0	1.44	0.658	0.428	0.391
N condensate + acid, % of N intake ³	1.9	1.4	1.4	1.2	0.22	0.202	0.252	0.601
Energy balance								
GE intake, ⁴ kJ/kg of BW ^{0.75} per day	2,480	2,537	2,517	2,544	130.7	0.661	0.409	0.771
ME intake, ⁵ kJ/kg of BW ^{0.75} per day	1,439	1,495	1,482	1,495	58.6	0.582	0.382	0.576
ME intake, % of GE	58.1	58.9	58.9	58.7	0.64	0.474	0.646	0.348
Energy in CH ₄ , kJ/kg of BW ^{0.75} per day	164	163	166	170	6.8	0.062	0.714	0.284
Energy in CH ₄ , % of GE	6.6	6.5	6.6	6.7	0.16	0.105	0.328	0.048
Energy in milk, kJ/kg of BW ^{0.75} per day	589	589	601	595	20.9	0.252	0.869	0.006
Energy in milk, % of GE	23.8	23.3	24.3	23.6	1.15	0.433	0.667	0.838
Heat production, kJ/kg of BW ^{0.75} per day	869	859	875	889	26.5	0.051	0.877	0.139
Heat production, % of GE intake	35.2	34.3	35.2	35.3	1.01	0.264	0.356	0.293
Energy retained, kJ/kg of BW ^{0.75} per day								
Total ⁶	-14	51	58	53	36.6	0.154	0.236	0.174
Protein ⁷	59	73	64	61	10.1	0.670	0.497	0.323
Fat ⁸	-74	-22	-5	-8	33.1	0.123	0.337	0.283

¹GS = grass silage (mainly ryegrass) with no inoculant; GS+S = grass silage with a short-term inoculant (applied 16 h before feeding); GS+L = grass silage with a long-term inoculant (applied at harvest); GS+S+L = grass silage with both long- and short-term inoculants.

²N retained = N intake – N in manure – N in milk – N in condensate collected from heat exchanger – N trapped from the outflowing air.

³N in condensate collected from heat exchanger plus N trapped in outgoing air.

⁴GE = gross energy.

⁵ME intake = GE intake – energy in excreta – energy in CH₄.

⁶Energy retention total = ME intake – heat production – energy in milk.

⁷Energy retention protein = (N retained/1,000) × 6.25 × 23.6 kJ/g.

⁸Energy retention fat = energy retention total – energy retention protein.

a lack of effect on DMI in the present study, no significant effect was observed here on NDF digestibility either. The long-term inoculant and grass silage used in the present study were the same as the grass silage (bales) and LM1 inoculant mixture used in Ellis et al. (2016) (experiment 3). In that in vitro study, LM1 significantly increased OM digestibility, as measured by OM disappearance during 72 h. Unfortunately, OM digestibility could not be reported in the current study because of total mixing of the urine and feces in the CRC, but no other results suggested such an OM digestibility increase occurred in vivo either (lack of effects on NDF digestibility, fat digestibility, DMI, and milk yield). Many differences likely exist between the in vitro and in vivo environment, including retention time. Within the rumen, degradation and passage of nutrients are occurring simultaneously. Because fractional passage rates vary between 0.02 and 0.05/h for

grass and corn silage (Warner et al., 2014), retention times are well below the 72-h endpoint used with in vitro studies. The longer incubation time in vitro may explain the significant effects, but observed effects on digestibility in vivo are lacking.

In the literature, increases in milk yield with silage inoculations are generally attributed to increases in ME intake, either due to increased DMI (Gordon, 1989; Mayne, 1990; Kung et al., 1993) or improved DM digestibility (Martinsson, 1992). However, in many studies changes in milk yield did not occur (Keady and Murphy, 1996, 1997; Kristensen et al., 2010) or were at nonsignificant levels (Bayatkouhsar et al., 2011). The results of the present study (nonsignificant effect on DMI, trend for greater milk yield) therefore fall within the range of results presented in the literature. Bayatkouhsar et al. (2011), for example, reported a significant increase in DMI with a numerical increase in milk yield

Table 6. Milk fatty acid (FA) composition (g/100 g of FA) from cows fed diets with or without long-term and short-term silage inoculation with lactic acid bacteria

Fatty acid	Treatment diet ¹				SEM	P-value		
	GS	GS+S	GS+L	GS+S+L		Long-term	Short-term	Long-term × short-term
C4:0	4.24	4.20	4.13	4.15	0.077	0.244	0.876	0.655
C6:0	2.43	2.44	2.42	2.44	0.090	0.828	0.493	0.890
C8:0	1.16	1.18	1.17	1.2	0.072	0.515	0.277	0.842
C10:0	2.38	2.44	2.46	2.53	0.209	0.235	0.342	0.985
C10:1 <i>cis</i> -9	0.26	0.27	0.27	0.28	0.024	0.447	0.364	0.759
C12:0	2.79	2.88	2.92	3.02	0.255	0.115	0.253	0.927
C12:1 <i>cis</i> -9	0.07	0.07	0.08	0.08	0.010	0.036	0.646	0.646
C13:0	0.07	0.07	0.07	0.07	0.007	0.081	0.836	0.836
C14:0	10.13	10.33	10.38	10.62	0.556	0.242	0.337	0.916
C14:0 <i>iso</i>	0.10	0.11	0.10	0.10	0.004	0.619	0.064	0.327
C14:1 <i>cis</i> -9	0.94	0.95	0.97	0.97	0.071	0.172	0.620	0.723
C15:0	1.14	1.18	1.16	1.19	0.054	0.619	0.241	0.868
C15:0 <i>iso</i>	0.24	0.24	0.24	0.25	0.011	0.854	0.583	0.854
C15:0 <i>anteiso</i>	0.39	0.40	0.40	0.39	0.017	0.679	0.890	0.343
C16:0	30.75	30.77	31.79	31.68	0.992	0.002	0.875	0.794
C16:0 <i>iso</i>	0.20	0.19	0.19	0.19	0.006	0.180	0.899	0.180
C16:1 <i>cis</i> -9	1.83	1.76	1.83	1.77	0.118	0.886	0.298	0.935
C16:1 <i>trans</i> -9	0.20	0.20	0.18	0.19	0.011	0.106	0.297	0.670
C17:0	0.69	0.69	0.67	0.67	0.016	0.037	0.418	0.870
C17:0 <i>iso</i>	0.37	0.36	0.37	0.37	0.018	0.708	0.272	0.272
C17:0 <i>anteiso</i>	0.37	0.36	0.36	0.36	0.010	0.146	0.237	0.546
C17:1 <i>cis</i> -9	0.32	0.31	0.30	0.30	0.031	0.099	0.562	0.642
C18:0	9.41	9.51	9.17	9.22	0.261	0.066	0.595	0.863
C18:1 <i>cis</i> -9/C18:1 <i>trans</i> -12 ²	21.36	20.91	20.47	20.04	1.760	0.097	0.389	0.989
C18:1 <i>cis</i> -12	0.11	0.11	0.11	0.10	0.006	0.080	0.020	0.709
C18:1 <i>cis</i> -13	0.14	0.13	0.14	0.13	0.010	0.804	0.041	0.804
C18:1 <i>trans</i> -10	0.13	0.13	0.14	0.14	0.005	0.048	0.324	0.667
C18:1 <i>trans</i> -11	1.26	1.24	1.18	1.18	0.059	0.008	0.729	0.604
C18:1 <i>trans</i> -15/C18:1 <i>cis</i> -11	0.70	0.68	0.66	0.65	0.071	0.083	0.311	0.659
C18:1 <i>trans</i> -6	0.19	0.19	0.19	0.19	0.005	0.115	0.813	0.813
C18:1 <i>trans</i> -9	0.15	0.15	0.15	0.15	0.005	1.000	1.000	1.000
C18:2 <i>cis</i> -9, <i>trans</i> -11	0.59	0.58	0.57	0.55	0.035	0.062	0.269	0.706
C18:2 <i>cis</i> -9,12	1.23	1.19	1.15	1.19	0.064	0.063	0.953	0.063
C18:3 <i>cis</i> -6,9,12	0.06	0.06	0.06	0.06	0.002	1.000	0.055	1.000
C18:3 <i>cis</i> -9,12,15	0.48	0.48	0.44	0.45	0.030	0.003	0.783	0.415
C20:0	0.15	0.16	0.15	0.15	0.005	0.249	0.021	0.813
C20:1 <i>cis</i> -11	0.06	0.07	0.07	0.06	0.005	1.000	0.626	0.069
C-20:2 <i>cis</i> -11,14	0.04	0.05	0.05	0.05	0.002	0.474	0.474	0.474
C20:3 <i>cis</i> -8,11,14	0.05	0.06	0.06	0.06	0.005	1.000	0.089	0.373
C20:4 <i>cis</i> -5,8,11,14	0.08	0.08	0.08	0.08	0.003	1.000	1.000	0.337
C-20:4 <i>cis</i> -8,11,14,17	0.04	0.04	0.04	0.04	0.003	0.610	0.610	0.153
C20:5 <i>cis</i> -5,8,11,14,17	0.06	0.07	0.06	0.07	0.002	0.785	0.416	0.785
C22:0	0.08	0.08	0.08	0.08	0.003	0.126	0.594	0.126
C22:5 <i>cis</i> -7,10,13,16,19	0.08	0.08	0.08	0.08	0.002	0.792	0.792	0.792
C24:0	0.05	0.05	0.05	0.05	0.002	0.552	0.552	0.244
Phytanic acid	0.33	0.34	0.33	0.33	0.021	0.708	0.778	0.708
Pristanic acid	0.04	0.04	0.04	0.04	0.003	0.210	0.414	0.767
Summary								
SFA	67.88	68.02	68.61	69.09	1.922	0.118	0.576	0.758
UFA	30.42	29.83	29.31	28.83	1.953	0.080	0.354	0.914
MUFA	27.71	27.16	26.74	26.22	1.888	0.102	0.341	0.974
PUFA	2.71	2.67	2.57	2.62	0.112	0.007	0.853	0.172
C18:1 <i>cis</i> and <i>trans</i>	24.03	23.53	23.04	22.57	1.825	0.071	0.344	0.967
Odd and branched-chain FA	4.26	4.29	4.29	4.26	0.053	0.440	0.489	0.986
Unidentified FA	2.15	2.15	2.08	2.08	0.055	0.038	0.932	0.843

¹GS = grass silage (mainly ryegrass) with no inoculant; GS+S = grass silage with a short-term inoculant (applied 16 h before feeding); GS+L = grass silage with a long-term inoculant (applied at harvest); GS+S+L = grass silage with both long- and short-term inoculants.

²C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12 because these 2 FA could not be separated in analysis. The portion of C18:1 *trans*-12 is considered negligible because it is always present in small quantities.

Table 7. Blood metabolite results from cows fed diets with or without long-term and short-term silage inoculation with lactic acid bacteria

Metabolite	Treatment diet ¹					P-value		
	GS	GS+S	GS+L	GS+S+L	SEM	Long-term	Short-term	Long-term × short-term
Glucose, mmol/L	3.34	3.26	3.14	3.40	0.116	0.774	0.395	0.138
Nonesterified fatty acids, mmol/L	0.23	0.18	0.19	0.16	0.053	0.432	0.370	0.828
BHB, mmol/L	0.58	0.52	0.68	0.58	0.096	0.214	0.221	0.736
Insulin, mIU/L	3.75	4.63	4.00	4.00	1.180	0.862	0.686	0.686

¹GS = grass silage (mainly ryegrass) with no inoculant; GS+S = grass silage with a short-term inoculant (applied 16 h before feeding); GS+L = grass silage with a long-term inoculant (applied at harvest); GS+S+L = grass silage with both long- and short-term inoculants.

with a commercial or laboratory-grown LAB inoculant applied on corn silage, but no treatment effect on composition of the silages, digestibility, ruminal pH, ruminal ammonia concentration, blood plasma metabolites, or milk composition were observed. Some of the differences observed in the literature may be attributable to the type of silage used. Although it is not pertinent to explaining the results of the present study, Ellis et al. (2016) observed in vitro an effect of LAB on the fermentation of grass or grass-clover silages, but not on fermentation of corn silage. This difference is likely due to LAB substrate preferences and the difference in silage chemical composition (mainly starch and sugar content) between grass and corn silage.

In the present study, the long-term LAB inoculant had significant effects on milk FA composition, but results contrast with those of Maragkoudakis et al. (2010), who found a significant increase in PUFA with a 12 log cfu/d treatment of *Lb. plantarum* to goats fed diets based on oat hay and barley straw. Maragkoudakis et al. (2010) also saw significant increases in C18:2 *cis*-9,12, C18:3 *cis*-9,12,15, and C18:2 *cis*-9,*trans*-11, whereas these FA were decreased in the present study with long-term LAB inoculation ($P = 0.063$, 0.003, and 0.062, respectively), suggesting an increase in ruminal biohydrogenation. Rumen biohydrogenation is affected by various factors, including the amount of UFA and

the amount of fiber in the diet (Harfoot and Hazlewood, 1997). The SFA and UFA contents of the control and treated grass silages were similar, however (Table 2), and NDF and fat digestibilities were also similar (Table 5).

Fibrolytic bacteria may be the main biohydrogenators in the rumen (Boeckaert et al., 2008), which would explain the correlation between biohydrogenation level and the fiber content of the diet. Diets low in fiber generally appear to result in rumen biohydrogenation of UFA that is reduced, incomplete, or both, and high fiber levels are associated with high levels of biohydrogenation in the rumen. Far less seems to be known about the effect of the type of fiber on biohydrogenation. A possible hypothesis is that high degradability of fiber coincides with high activity of fibrolytic microbes and consequently with high biohydrogenation activity. Li and Meng (2006) evaluated the effect of different sources of fiber (wheat straw, soybean hulls, purified cellulose) on biohydrogenation of unsaturated fatty acids in vitro. The more lignified, slowly degradable fiber of wheat straw was associated with reduced biohydrogenation levels of unsaturated C18 FA compared with the more rapidly degradable fiber of soybean hulls or the purified source. These results indicate that next to the level of fiber, the type of fiber also affects rumen biohydrogenation.

Table 8. Milk microbial analysis (cycle threshold values) from cows fed diets with or without long-term and short-term silage inoculation with lactic acid bacteria

Variable	Treatment diet ¹					P-value		
	GS	GS+S	GS+L	GS+S+L	SEM	Long-term	Short-term	Long-term × short-term
Milk pathogen								
<i>Klebsiella</i> spp.	40	40	40	40	—	—	—	—
<i>Serratia marcescens</i>	36	36	37	36	1.0	0.809	1.000	0.305
<i>Arcanobacterium pyogenes</i> / <i>Peptoniphilus indolicus</i>	37	37	37	37	0.5	0.461	0.766	0.656
Yeast	40	40	40	40	—	—	—	—
Total pathogen	153	153	154	153	1.3	0.663	0.709	0.755

¹GS = grass silage (mainly ryegrass) with no inoculant; GS+S = grass silage with a short-term inoculant (applied 16 h before feeding); GS+L = grass silage with a long-term inoculant (applied at harvest); GS+S+L = grass silage with both long- and short-term inoculants.

The milk FA profile has been suggested to be a proxy of CH₄ production (Chilliard et al., 2009). Although the significantly increased level of C16:0 and decreased level of C18:1 *trans*-10 plus C18:1 *trans*-11 in milk fat with long-term LAB inoculation would suggest increased CH₄ production (g/kg of DM and g/kg of FPCM; van Lingen et al., 2014), this pattern was not observed in the present experiment.

Milk Microbial Analysis

Mastitis usually has an infectious origin through introduction of bacteria via the teat duct. Traditionally *Staphylococcus aureus* has been the main cause, but *Staphylococcus epidermidis* is also emerging as a leading cause (Zhang and Maddox, 2000). Soleimani et al. (2010) showed an inhibitory effect of LAB inoculation with *Lb. plantarum*, but not of *Lb. acidophilus*, *Lb. casei*, and *Lb. reuteri*, on growth of *S. aureus* in vitro and suggested that it might be used to control *S. aureus* with mastitis. Jiménez et al. (2008) examined the effect of orally supplemented *Lactobacillus salivarius* and *Lactobacillus gasseri* on staphylococcal mastitis in humans, and found that although mastitis persisted in the control groups, the LAB treatment group showed no clinical signs of mastitis by d 14. Elsewhere, *Lb. gasseri*, *Lb. fermentum*, and *Lb. salivarius* isolated from milk have shown a probiotic effect in controlling mastitis (Martín et al., 2005).

How orally supplemented LAB may reach the mammary gland has been up for some debate (via a direct pathway or contamination) (Fernández et al., 2013). Either way, it was of interest to examine whether LAB inoculation as used in the present study had any effect on several microbial species associated with mastitis in milk, as well as the milk SCC, a typical indicator of the presence of mastitis. However, of the mastitis pathogens analyzed in milk, *Klebsiella* spp. and yeast were negligible (Table 8). *Serratia marcescens* and *Arcanobacterium pyogenes/Peptoniphilus indolicus* were present in measurable quantities, but their Ct count numbers were low and did not differ between treatments (Table 8). Milk SCC did not differ significantly between treatments either, although it was numerically lower with the short-term LAB inoculation (Table 4).

As expected, milk SCC and microbial Ct values were correlated. Regressions of total pathogen load (Ct) = 155 (±1.8) – 0.013(±0.0036) × milk SCC (cells × 1,000/mL) (slope *P* = 0.001) and *Serratia marcescens* (Ct) = 36 (±1.6) – 0.0092 (±0.0032) × milk SCC (cells × 1,000/mL) (slope *P* = 0.008) both produced significant negative slope parameters. Overall, no indication of mastitis inhibition was apparent, but the control treatment cows had minimal presence of mastitis pathogens

to start with. Without first inducing mastitis, making conclusions about any inhibitory effect by LAB is difficult, and it therefore remains an interesting potential implication of LAB inoculants.

Methane Emissions

In the current study, no significant effects of long- or short-term LAB inoculation were observed on CH₄ emission regardless of the unit of expression (Table 4), except for a trend of increased CH₄ produced per unit of metabolic BW (*P* = 0.062; Table 5). This trend appears to be related to a numerically greater energy intake and numerically greater NDF digestibility. This is despite several in vitro studies having suggested that reductions in CH₄ emissions are possible because of either changes in OM digestibility or direct antimicrobial effects of LAB. For example, O'Brien et al. (2013) reported that cumulative CH₄ (mL/24 h) and CH₄/CO₂ (mol/mol) were reduced with a *Lb. plantarum* (TUA1490L) inoculum or a cell-free supernatant during an in vitro incubation. However, total VFA production (mM) was also significantly reduced, and cumulative CO₂ (mL/24 h) was numerically reduced, indicating a general decline in fermentation. Asa et al. (2010) investigated in vitro fermentation of a klesingrass-concentrate mixture with supernatant from *Lc. lactis* (ATCC 19435), *Lc. lactis* (NCIMB702054), *Lb. plantarum* (TUA1490L), and *Leuconostoc citreum* (JCM 9698). The *Lb. plantarum* (TUA1490L) supernatant significantly reduced cumulative CH₄ production (mL), but also numerically reduced total VFA concentration (mM) and total CO₂ production (mL). Jalc et al. (2009a) investigated inoculation of grass silage in vitro with *E. faecium* (CCM 4231), *Lactobacillus fermentum*, or *Lb. plantarum* (CCM 4000) and found significant reductions in CH₄ (mol of CH₄/kg of digested DM) for all LAB. For the *E. faecium* and *Lb. fermentum* inoculations, OM degradability (g/kg of DM) was numerically reduced, but for *Lb. plantarum* it was significantly increased, which seemed promising. Jalc et al. (2009b) then investigated the same LAB on corn silage in vitro and found significant increases in OM degradability (g/kg of DM) with all 3 LAB inoculations and no significant changes in CH₄ (mol/kg of digested DM). In our own previous in vitro study, LM1 from experiment 3 (Ellis et al., 2016), involving the same grass and long-term inoculant as in the present in vivo study, resulted in increased CH₄ (mL/g of OM) at early time points (up to 24 h) which was associated with an increase in total gas production and OM digestibility. These in vitro results did not translate into similar responses in the present in vivo experiment, however.

These results illustrate the strain and substrate specificity of LAB on in vitro fermentation, and they show that similar effects may not be obtained in vivo and in vitro. To our knowledge, no other published work has examined LAB inoculants for their effect on CH₄ emissions in vivo. With the variability between strains and substrates, and with some LAB appearing to increase digestibility and others appearing to decrease it or keep it the same, the challenge may be to find the correct LAB for application in vivo. In the present study, because of minimal changes in DMI, digestibility, CH₄ and milk yield, such changes did not occur, nor was any direct antimethanogen effect in the rumen (short-term inoculant) indicated.

Lack of Significant Long-Term Inoculum Effects

The present results indicate minimal responses in animal performance to long-term inoculation of grass silage with LAB. The cause of significant improvements in animal performance observed in some studies, but not others, is still unclear. Strain differences, compounded with differences in basal silages, silage characteristics, and rumen fermentation conditions, are likely responsible for the varying results in the literature.

Based on a power analysis according to the procedure outlined in Kononoff and Hanford (2006), the experimental design and number of animals used in this study should be sensitive enough to detect significant differences (in milk yield, for example) of ~10% with a probability of 0.969. Supporting this assumption, others that used the same experimental design and number of animals detected significant effects within the range of 9 to 12% difference (Martin et al., 2008; Chilliard et al., 2009). Therefore, it does not seem likely that the lack of significant effects observed in the present study was due to an insufficient number of animals, but rather was due to a true lack of treatment effect.

However, several possible reasons exist for the lack of effect of the long-term inoculant treatment observed in the present study that can be examined, related to either failure of inoculated LAB to dominate fermentation or failure to inhibit adverse microbial activity (Weinberg and Muck, 1996). The high DM, high pH, and low LA concentrations of the silages in the present study suggest restricted fermentation and a relatively poor ensiling, which may have contributed to the lack of significant treatment effects. However, the ensiling parameters are fairly typical for the Netherlands where the average for grass silage was 432 g/kg of DM, pH 5.8, LA 39 g/kg of DM, and acetate 12 g/kg of DM in the year of study, 2013 (BLGG AgroXpertus, Oosterbeek, the Netherlands). From Table 1, the average LA:acetate ratio was 3.5 for the control silage and

3.1 for the treated silage, which indicated small differences existed in fermentation end products and that LA as an end product did not dominate fermentation in the treated silage. However, the long-term inoculant also included a *L. buchneri* strain, a heterofermentative LAB that grows more slowly and produces a greater concentration of acetic acid. Consequently, changes in the LAB population of the silage may not have been overtly reflected in large changes in the LA content or LA:acetate ratio. It is also possible that the inoculant strains did not grow in the grass silage as well as desired or that no results were found because of technical problems related to LAB not being viable at the time of application (e.g., too long storage, heat damage), uneven mixing of the inoculant in the forage, low ambient temperatures, or slow bailing. However, analysis of control and treated silage bales at the time of the trial showed an average total LAB of 6.41 (± 0.23) and 7.02 (± 0.19) log cfu/g, for the control and treated silage, respectively, indicating that treated silage was indeed greater in LAB content. These values were also greater than the LAB count on fresh grass, prebaling (5.94 ± 0.03 log cfu/g). This finding supports the premise that the LAB applied were viable and able to grow and ferment during ensiling. It also supports the premise that the eventual LA content, the LA:acetate ratio, or both may not be representative of the LAB mixtures' ability to dominate fermentation or the LAB count in silages. Additionally, if the control silage already has high LAB counts naturally, then the inoculant may not provide any additional benefit, which may explain the lack of treatment effect.

The LAB strains present in the current inoculations were isolated from tomato pulp (*Lb. buchneri* LB1819) and maize silage (*Lb. plantarum* 6072). Hill (1989) isolated strains of *Lb. plantarum* from corn, lucerne, and sorghum and found that when these 3 strains were co-inoculated back onto the crops, the strain originally isolated from a specific crop was found in greater numbers and thus dominated in the silage of that crop. This finding suggests that the best strains for a specific crop would be those obtained from that crop originally. However, characteristics of individual LAB strains can vary considerably and may be beneficial on various other crops. The strains and strain combinations used in the present study were selected because they were commercially available and had been previously shown to have beneficial effects on silage preservation and quality. The *Lb. plantarum* strain used is generally known to result in rapid formation of LA, decreasing silage pH rapidly and preventing development of clostridia, yeasts, fungi, and molds. The *Lb. buchneri* strain used produces acetic acid and is known to inhibit fungi and help preserve silage upon exposure to air. Previous

in vitro work (Ellis et al., 2016) showed that inoculants of *Lc. lactis*, *Lb. plantarum*, and *Lb. buchneri* showed the most promise for effects on OM digestibility and CH₄ mitigation in grass silage, which indicates potential for in vivo mitigation. A *Lc. lactis* strain was used in both short- and long-term inoculants. The specific strain used for the long-term inoculant has an oxygen-scavenging ability that may play an important role in ensuring a fast establishment of anaerobic conditions in the silage. However, the specific *Lc. lactis* strain used for the short-term inoculation has been shown to produce nisin, which acts as an antimicrobial and has been shown to reduce CH₄ during in vitro studies (Asa et al., 2010).

Failure to inhibit adverse microbial activity may also contribute to a lack of positive treatment effect. Two types of microbial activity can be problematic during silage making, clostridial growth and aerobic microbes. In low DM silages, low WSC content, high buffering capacity, or both may prevent efficient LAB fermentation and thus the development of a sufficiently low pH to prevent clostridial activity (Hellings et al., 1985; Haigh et al., 1987). The DM content of silages used in this trial were on the high side, however (Table 1), and the reducing sugar content adequate (Table 1), so clostridial activity is unlikely to explain the absence of beneficial effects here. Regarding aerobic stability, some studies have found that homofermentative LAB inoculants actually enhance aerobic deterioration in cereal silages (Weinberg et al., 1993). The explanation given is that high levels of residual WSC, combined with high LA concentrations and lack of inhibitory VFA (such as acetic, propionic, and butyric acids), contributed to aerobic spoilage because WSC are substrates for molds and yeasts and VFA inhibit these organisms (Weinberg et al., 1993). In the present trial, however, the average total yeast count was 3.55 (± 0.59) for control silage and 1.15 (± 0.84) for treated silage (log cfu/g), and mold was 1.94 (± 0.86) for control silage and 0.5 (± 0.5) for treated silage (log cfu/g). This finding suggests that additional spoilage due to the LAB inoculation was not a problem in the present trial. Indeed, *Lb. buchneri*, 1 of the 3 microbes used in the long-term LAB inoculant may have preventative effects on aerobic spoilage because it is heterofermentative, produces acetic acid, and also converts LA to acetic acid when oxygen is present (Weinberg and Muck, 1996). With this second mechanism, upon exposure to oxygen approximately one-third of LA will be lost as CO₂ on a mass basis. Such a small loss of DM (1–2%) is much less, however, than the potential loss due to aerobic microbes (Weinberg and Muck, 1996). It seems unlikely that this loss was occurring in the silages used in the present study. Therefore, it seems unlikely that the lack of treatment

effect was due to the inability of LAB inoculant to dominate fermentation or to spoilage.

Lack of Significant Short-Term Inoculant Effects

The LAB have been suggested to have a direct probiotic effect in either silage or the rumen. In a meta-analysis, Muck (1993) noted improvements in fiber digestibility in 30% of the trials in which it was measured. This result is curious because LAB are not known to have any direct activity on polysaccharides (Weinberg and Muck, 1996). This outcome may have occurred indirectly, however, through an increase in protein availability and digestibility in silage, with the result being beneficial to fibrolytic bacterial growth in the rumen. Alternatively, a probiotic effect has been proposed via the ability of LAB to inhibit pathogens, the ability of some LAB to produce a variety of antimicrobial substances such as bacteriocins, or both (Vandenbergh, 1993; Muller et al., 1996). These compounds may inhibit detrimental microbes in the silage or a particular group of microbes in the rumen. Typically, LAB are not prevalent within the rumen of fresh forage-fed animals, and so their inoculation into the rumen via silage represents a somewhat novel scenario to the rumen with that substrate. The LAB become more prevalent in the rumen when large amounts of sugar or starch are fed (Van Soest, 1994) because of their affinity for these substrates and tolerance for low rumen pH. Weinberg et al. (2003) found that addition of glucose with LAB markedly enhanced the survival of inoculated LAB in rumen fluid, confirming that the LAB strains could compete effectively with the ruminal microflora for exogenous glucose, although some strains grew better than others. Although plenty of substrate exists in the concentrate-fed rumen for rapid LAB growth, a proportion of the dietary WSC has already been metabolized by silage microbes in the rumen of silage-fed animals, meaning that silage-fed animals consume less WSC and the LAB present in the rumen may grow less rapidly because of low substrate availability. In our previous in vitro study (Ellis et al., 2016), 10 LAB strains were added directly to the in vitro medium in 2 experiments to examine their probiotic effects on OM digestibility, cumulative gas, and CH₄ production. Addition of one strain resulted in a significant increase in OM digestibility (*Lb. plantarum*), and the gas or CH₄ production was not affected by any strain. This finding suggests that LAB potentially have a direct probiotic effect, but strain specificity certainly exists and the mode of action also needs to be determined. The *Lc. lactis* strain used in the short-term inoculant in the present study has been shown to produce nisin, a substance with antimicrobial activity that has been shown

to inhibit CH₄ production in vitro (Asa et al., 2010). However, in the current study, no effect of the short-term inoculant was found on any response variable, and others have also reported mixed results. Keady and Steen (1996) found that a short-term inoculation of grass silage with *Lb. plantarum* did not significantly affect DMI, total-tract digestibility, or degradability or fermentation in the rumen, and they found no evidence of a “direct” effect of the LAB in the rumen. However, in a previous study by the same authors, a long-term inoculation with *Lb. plantarum* MTD1 was associated with significant increases in DM, OM, N, and NDF digestibility coefficients with inoculated silages (Keady and Steen, 1995). Khuntia and Chaudhary (2002) also found that a short-term inoculation with a mixed culture of LAB increased DMI, live weight gain, and DM digestibility in calves. It appears this area could benefit from further research, to determine if and under what conditions such a probiotic effect occurs and what LAB strains to use to attain this effect.

CONCLUSIONS

Results of this study indicate minimal responses in animal performance to both long- and short-term inoculation of grass silage with LAB. Dry matter intake was not affected by long- or short-term silage inoculation, nor was dietary NDF or fat digestibility or N or energy balance. Milk composition (except urea) and FPCM were not affected by long- or short-term silage inoculation, nor was milk microbial count. However, milk yield tended to be greater with long-term silage inoculation. As a result, CH₄ expressed in units of grams per day, grams per kilogram of DMI, grams per kilogram of milk, or grams per kilogram of FPCM was not affected by long- or short-term silage inoculation. However, CH₄ expressed in units of kilojoules per kilogram of BW^{0.75} per day tended to be greater with long-term silage inoculation. Strain and dose differences, compounded with different basal silages and rumen fermentation conditions, are likely responsible for the lack of significant treatment effects in the present study, although positive effects have been observed by others. Analysis of the complex interrelationships between experimental factors and cow responses would benefit from a thorough meta-analysis of the available literature to discern under what conditions a changed response can be anticipated. Ideally, such analysis would be done before further in vivo testing.

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