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In vivo changes in rumen fermentation and bacterial profiles after administering antibiotic resistant bacterium (*Lactococcus lactis*) as rumen modifier in Holstein lactating dairy cows facing ruminal acidosis challenge

Wedad Yahfoufi, Mohsen Danesh Mesgaran, Mohammad Hadi Sekhavati, Ali Reza Vakili, Mojtaba Tahmoorespur*

Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

*Corresponding author,
E-mail address:
danesh@um.ac.ir

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ORCID
Wedad Yahfoufi
0009-0001-5614-0286
Mohsen Danesh Mesgaran
0000-0002-2738-5284
Mohammad Hadi Sekhavati
0000-0001-8091-3798
Ali Reza Vakili
0000-0001-7862-9763
Mojtaba Tahmoorespur
0000-0002-5443-7529

Abstract The objective of this study was to evaluate the effect of an antibiotic resistant bacterium (*Lactococcus lactis; L. lactis*) on nutrient apparent digestibility, fermentation parameters, ruminal pH and ammonia nitrogen, productivity and ruminal bacterial abundance in multiparous lactating Holstein cows during ruminal acidosis challenge. Four rumen-fistulated Holstein dairy cows were assigned to the following treatments as: 1) basal diet without any additive (CON) and 2) basal diet inoculated with 1×10^{11} cfu d^{-1} transgenic *L. lactis* (BACT) which was infused into the rumen for two consecutive periods each consisting of seven days and separated by 10 days recovery. During the study, ruminal acidosis was induced by direct introduction of ground corn grain and whey powder into the rumen (3 kg DM per head per d) for 4 days. Milk fat content was increased in cows inoculated with BACT ($P<0.05$). Cows treated with BACT had higher apparent digestibility of dry matter, crude protein, neutral detergent fiber, acid detergent fiber and ether extract. Both ruminal pH and ammonia nitrogen concentration did not show significant responses to the experimental treatments, while their pattern related to the sampling time, for 8 h after BACT inoculation, was significant ($P<0.05$). Moreover, bacterial-treated group exhibited an increase in total ruminal volatile fatty acid production and molar concentration of acetate ($P<0.05$). *L. lactis* inoculation increased ($P<0.05$) the abundance of lactic acid utilizing (*M. elsdenii*) and cellulolytic (*R. flavefaciens*) bacteria. Our results demonstrated that ruminal inoculation with the antibiotic resistant bacterium *L. lactis* might improve rumen fermentation pattern, as seen in acetate concentration, and change bacterial population in benefit of hydrogen consuming bacteria during ruminal acidosis.

Keywords: acidosis, cow, fermentation, transgenic *Lactococcus lactis*

Introduction

Ruminal acidosis is a digestive disorder caused by the sudden intake of easily digestible carbohydrates, primarily cereal grains (Aschenbach et al., 2011). Rapid degradation of cereal starch in the rumen leads to the accumulation of organic acids and volatile fatty acids (VFAs), which can exceed the rumen's buffering capacity, resulting in a decrease in pH. This condition increases the incidence of ruminal acidosis which is associated with diminished feed intake, liver abscesses (Nagaraja and Lechtenberg, 2007), milk fat depression (Kleen et al., 2003), and laminitis (Lean et al., 2013). One of the strategies in mitigating such disorders is application of direct-fed microbials (DFM), viable organisms that provide beneficial effects on animal health and performance as supplements (Arowolo et al., 2018). Several non-pathogenic microorganisms have been used as DFM such as lactic acid bacteria (LAB) of *Lactobacillus* spp., *Bifidobacterium,*

Enterococcus, Bacillus spp., lactate-utilizing bacteria (LUB) and various strains of yeast (Soe et al., 2010). *Lactococcus lactis* (*L. lactis*) is one of the most commonly used probiotic bacterial species in ruminants (Yirga, 2015). It is likely to be a transient bacterium that was introduced with the feed (Kung, 2006) and has been shown to improve feed efficiency, growth rate, and ruminal nutrient degradability in feedlot steers (Baah et al., 2009). Soe et al. (2010) reported four common modes of action of lactic acid-producing bacteria in ruminants which are: constant lactate supply, adaptation to lactic acid accumulation, stimulation of LUB, and pH stabilization. Antimicrobial peptides (AMPs) are among the most studied antibiotic alternatives (Li et al., 2018). They have been shown to improve growth performance, intestinal function, and nutrient digestibility in weanling pigs and broilers (Yoon et al., 2014; Wang et al., 2020b), inhibit methane production in cattle (Lee et al., 2002), treat mastitis in bovine (Cho et al., 2024), improve daily weight, rumen papillae diameter, the micropapillary density, and volatile fatty acid content in bulls (Shi et al., 2023). A subgroup of AMPs includes those derived from large proteins, such as those derived from the lactoferrin protein (lactoferrampin (LFA) + lactoferricin (LFC), termed camel Lactoferrampin chimera, cLFchimera (Brogden et al.,2005). The LFA and LFC are two rich sources of hydrophobic and cationic antimicrobial peptides of N-terminal lactoferrin protein with activity against a wide range of microorganisms (van Der Kraan et al., 2004). The cLF chimera was successfully expressed in *L. lactis* and exhibited bactericidal activity against various bacteria (Tanhaieian et al., 2018). This antibiotic resistant transgenic *Lactococcus lactis* (BACT), previously selected and an *in vitro* ruminal culture, was established to assess its effects on nutrient disappearance along with different dietary lactose/starch ratio, where positive effects on dry matter (DM) and crude protein (CP) disappearance were recorded (Yahfoufi et al., 2024). The objective of this study was to determine the *in vivo* effect of BACT, expressing cLF chimera, on apparently nutrients digestibility, ruminal fermentation parameters and bacterial abundance, milk yield and milk components in rumen fistulated lactating Holstein cows under ruminal acidosis condition.

Materials and methods

Animals and feeding

The experiment was conducted at the Research Farm of the Agricultural Faculty of Ferdowsi University of Mashhad, Iran. The experimental protocols were reviewed and approved by the Animal Care Committee of the University. Four multiparous Holstein lactating cows (BW= 660±35 kg, 160 days in milk (DIM), milk yield= 28±2.2 kg) fitted with rumen fistulae (10 cm; Bar Diamond Inc., Parma, ID), were assigned to 1 of 2 treatments in a replicated randomized block design for 7 days as the experimental period with 10 d as recovery phase. Animals were housed in tie stalls and had free access to fresh water and feed. Experimental treatments were a basal diet (CON; Table 1), and basal diet plus BACT inoculated directly into the rumen at a dosage of 5 mL of 10^{11} colony-forming unit (cfu)/mL of fresh live culture per head, repeated every day one hour before the morning feeding for 7 consecutive days. Ruminal acidosis was induced by intra-ruminal feeding of 3 kg DM of either corn grain meal (114 g/kg DM) or whey powder (127 g/kg DM) per cow per day at morning feeding in each experimental period (Figure 1). The BACT culture was prepared on M17 broth and preserved in 25% glycerol in an ultra-low temperature freezer. Prior to administering, the bacteria were revived in M17 broth. For suspension, the M17 broth was inoculated with 1% (v/v) BACT culture and incubated at 38.6°C overnight, using culture broth method (Bonnet et al., 2020). The basal diet was formulated to meet the requirements for crude protein, net energy for lactation (NEL), minerals and vitamins for a cow weighing 660 kg, producing 32 kg of 3.5% fat corrected milk (FCM) per day, and consuming 20 kg of DM/d (NRC, 2001). Dry matter intake (DMI) was recorded daily.

Table 1. Ingredients and nutrient composition of the basal diet

Ingredients (g/kg DM)	
Alfalfa hay	210
Corn silage	319
Straw	31
Cotton seed	129
Corn grain	182
Soybean meal	130
Vitamin and mineral premix[1]	1
Composition (% DM)	
Crude protein	16.8
Neutral detergent fiber	39.6
Acid detergent fiber	21.50
Ether extract	3.2
Non-fibrous carbohydrate	32
Ash	6.1
Starch	20.2

[1]Contained per kilogram: 975000 IU of vitamin A, 750000 IU of vitamin D3, 1,800 IU of vitamin E, 143.0 g of Zn, 76.0 g of Mn, 48.6 g of Cu, 19.5 g of Se, 18.4 g of Fe, 8.0 g of Ca, and 1.3 g of Co.

Sample collection

Offered feed and orts for each animal were weighed and sampled during each experimental period. Cows were milked 3 times a day at 0700, 1400, and 2100 h, with the milk yield recorded at each milking. On the last day of each experimental period, milk samples were collected from each cow during each milking and subsequently combined based on the average milk production (morning, afternoon, and night). These samples were preserved at room temperature using potassium dichromate. Fat-corrected milk (3.5%) was calculated using the formula: (0.432× kg of total daily milk) + (16.216× (kg of total daily fat) / 1000) (Sklan et al., 1992). Rumen fluid was collected from each cow on the last day of the experimental period at 0.0, 0.5, 1, 2, 3, 4, and 8 h after the morning feeding, with pH measured immediately using a glass electrode pH meter (Model 691; Metrohm, Herisau, Switzerland). Samples were

also taken and stored at – 20°C until analyzed for NH_3-N (5 mL mixed with 0.2 N HCl) and VFA (5 mL mixed with 1 mL of 25% (w/v) meta-phosphoric acid). Rumen content was also sampled at 8 h post-inoculation and immediately preserved at -80°C, which was then used to isolate ruminal bacteria, including L. *lactis*, R. *flavefaciens*, S. *bovis*, and *M. elsdenii*.

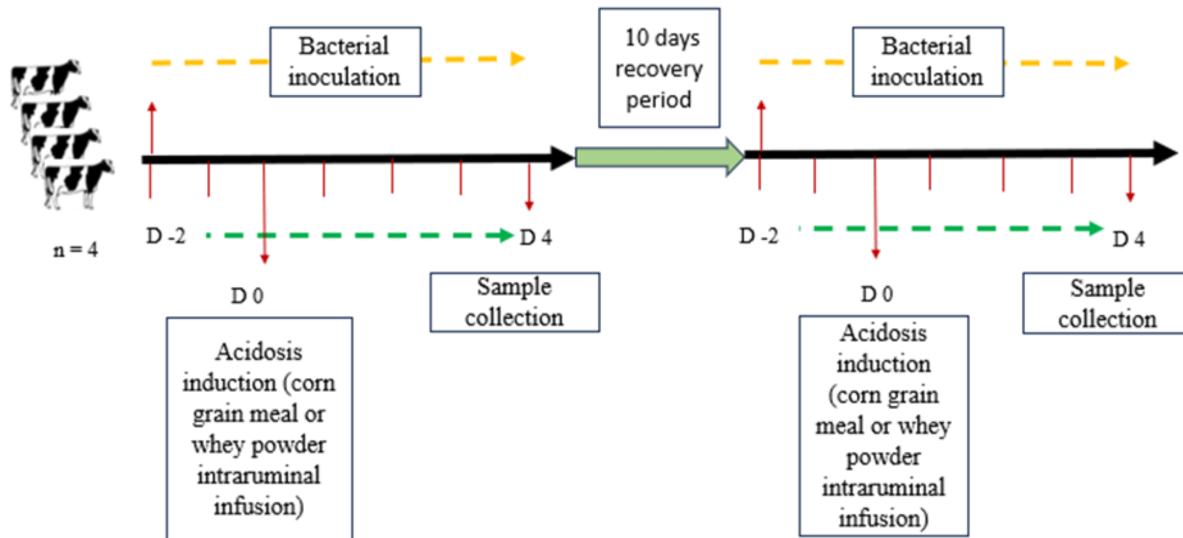

Figure 1. Diagram showing the stages of each experimental period.

Feed refusals for each cow were measured daily, and the total DMI was monitored by calculating the difference, assuming a similar DM content in both the feed offered and orts. Twelve hours post-morning feeding, samples of rectal feces were collected from each cow to evaluate fecal output, DM digestibility, and DMI. Samples of feed, residuals, and feces were individually dried in an air-draft oven at 60°C for 48 h until a constant weight was achieved (AOAC, 2007). Samples of feed ingredients, feed refusals, and fecal matter were dried in a forced-air oven at 60°C for 48 h and then ground to a particle size of 2 mm before analysis. Standard methods were used to measure dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and ether extract (EE) in the feed, orts, and fecal samples (AOAC, 2005).

Concentration of ruminal ammonia nitrogen (NH_3-N) was analyzed using the phenol hypochlorite method developed by Weatherburn (1967). Ruminal fluid samples for VFA analysis were thawed at room temperature and then centrifuged (Eppendorf AG, Hamburg, Germany) at 3000g for 20 min at 4°C. Concentrations of ruminal VFAs were measured with a gas chromatograph (YL6100 GC; Young Lin Instrument, Anyang, South Korea) equipped with a 50 mm × 0.32 mm silica-fused column (CP-Wax Chrompack Capillary Column; Varian, Palo Alto, CA, USA). Helium served as the carrier gas with initial and final oven temperatures set at 55 and 195°C respectively. The temperatures of the detector and injector were maintained at 250°C and crotonic acid (1:7 v/v) was used as the internal standard. Concentrations of fat, protein, lactose, and urea nitrogen (MUN) in milk samples were analyzed using Fourier-transform infrared spectroscopy (FT-IR; LactoSope TM FT-B milk analyzer, CombioScope FTIR 600 HP, Delta instruments Drachten, The Netherlands), and milk analyzer (Ekomilk® Horizon unlimited, Bulgaria).

DNA extraction and Real-time PCR

Microbial DNA in the sediment of the rumen fluid was extracted using the spin column method (Boom et al., 1990) with the FavorPrep™ Tissue Genomic DNA extraction Mini Kit. The DNA concentration was quantified using a Nanodrop spectrophotometer by measuring the absorbance at 260 nm. The DNA quality was assessed through gel electrophoresis of aliquots of the polymerase chain reaction (PCR) product in a 1% agarose gel with 1× TAE buffer. The extracted DNA was stored at −80°C for quantitative real-time PCR (qPCR) analysis. Quantitative real-time PCR was performed using Applied Biosystems StepOnePlus Real-Time PCR system, (Applied Biosystems Co., USA), to investigate the abundance of *M. elsdenii, S. bovis, R. flavefaciens, L. lactis* (Kubista et al., 2006). The total qPCR reaction volume of 14 µL comprised 7.5 µL of SYBR Green PCR Master Mix, 1 µL each of the forward and reverse primers, 3.5 µL of sterile water, and 2 µL of the extracted DNA sample from each treatment group. Species-specific PCR primers, used to amplify the 16S rRNA, were selected from the literature (Table 2). Amplification was performed with the following cycling parameters: 94°C for 5 min for denaturation, 45 cycles of 94°C for 30 s, 62°C for 30 s for annealing, 72°C for 20 s for extension, and 72°C for 10 min for elongation. Each reaction mixture was run in duplicate, and negative controls were loaded to screen for possible

contamination and dimer formation. The C_t (threshold cycle) values were determined during the exponential phase of amplification, and the mean C_T of triplicates for each sample was used for calculations.

Table 2. Oligonucleotide primers used for real-time PCR assay

Species	Primer	Sequence (5'→ 3')	Product size (base pair)	Reference
Megasphaera elsdenii	MegEls1-F	GACCGAAACTGCGATGCTAGA	129	Ouwerkerk et al. (2002)
	MegEls1-R	CGCCTCAGCGTCAGTTGTC		
Ruminococcus flavefaciens	RumFla-F	TCTGGAAACGGATGGTA	295	Koike and Kobaiashi, (2001)
	RumFla-R	CCTTTAAGACAGGAGTTTACA		
Lactococcus lactis	L.lactis-F	CTGCCTCCTCTCCCTAGTGC	500	Tanhaieian et al. (2018)
	L.lactis-R	CTAAGGATGATTTCTGGCAGGG		
Streptococcus bovis	StrBov-F	TTCCTAGAGATAGGAAGTTTCTTCGG	82	Stevenson and Weimer, (2007)
	StrBov-R	ATGATGGCAACTAACAATAGGGGT		

F forward, R reverse

Statistical analysis

The experiment was performed as a randomized block design based on the following model: $Y_{ij} = \mu + A_i + B_j + e_{ij}$, where Y_{ij} is the measured value, μ is the overall mean, A_i is the effect of treatment (i=2; CON and BACT), B_j is the block effect (corn grain meal or whey powder), and e_{ij} is the residual error. Data on pH and NH_3-N, obtained hourly, were analyzed using the repeated measures ANOVA according to the following model: $Y_{ij} = \mu + \alpha_i + \beta_j + e_{ij}$, where Y_{ij} is the response variable, μ is the overall mean of the response variable, α_i is the fixed effect of treatment (i=2; CON and BACT), β_j is the fixed effect of time (hours) (j= 7; 0, 0.5, 1, 2, 3, 4, 8) and e_{ij} is the random error term.

All data were analyzed using the PROC MIXED procedure in SAS (2004, version 9.4). Significant effects were declared at P<0.05, and trends were declared at 0.05<P<0.10. Differences among the treatments were analyzed using the Tukey-Kramer's multiple comparison procedure within the LSMEANS statement of SAS. Since we did not detect any significant effect of the block on the measured variables, it was not considered further in our results and discussion.

Results

Ruminal pH and fermentation parameters

Figures 2 and 3 illustrate the effects of bacterial supplementation on ruminal pH and NH_3-N concentration, respectively. The addition of BACT did not significantly affect ruminal pH (P>0.05); however, the BACT group displayed higher numerical values at the measured time points.

The ruminal NH_3-N concentration ranged from 10 to 15 mg/dL and was not affected by bacterial treatment. The effect of BACT inoculation on ruminal VFA concentrations and their molar proportions is presented in Table 3. Total VFA concentration significantly increased with bacterial treatment (109.81 *vs.* 100.22 m*M* in CON group). Individual VFA concentrations varied among treatments, with the BACT group exhibiting an increase in acetate molar concentration compared to the CON group (76.17 versus 64.45). Additionally, there was a tendency for increased concentrations of propionate, valerate, and iso-butyrate (0.05<P<0.1).

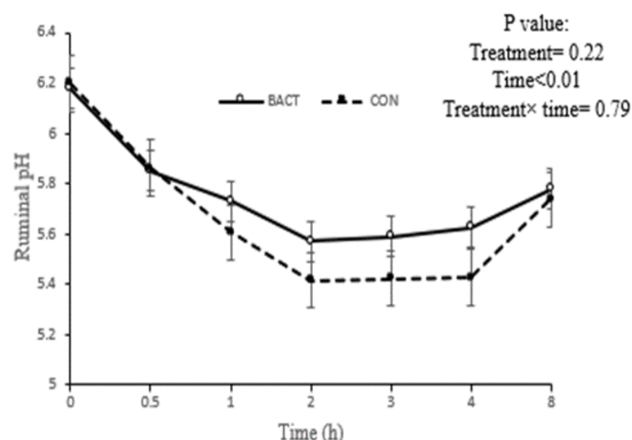

Figure 2. *In vivo* effect of inoculation of an antibiotic resistant bacterium (*Lactococcus lactis*, BACT) on ruminal pH pattern during 8 h after the morning feeding in rumen- fistulated lactating Holstein cows: Control (CON) *vs.* BACT (cfu= 1×10^{11}, BACT).

Dry matter intake (DMI), milk yield, milk composition

Data on the average DMI, milk yield, and milk composition are presented in Table 4. Supplementation with BACT had no significant effect on DMI, milk production, milk protein, or lactose content (P>0.05). Milk urea nitrogen (MUN) levels were not influenced by bacterial inoculation; however, the BACT group exhibited a higher milk fat content, increasing from 3.54% in the CON group to 3.8% in the BACT group. Similarly, milk fat yield was greater in the BACT group compared to the CON treatments (1.13 kg/d *vs.* 1.01 kg/d respectively).

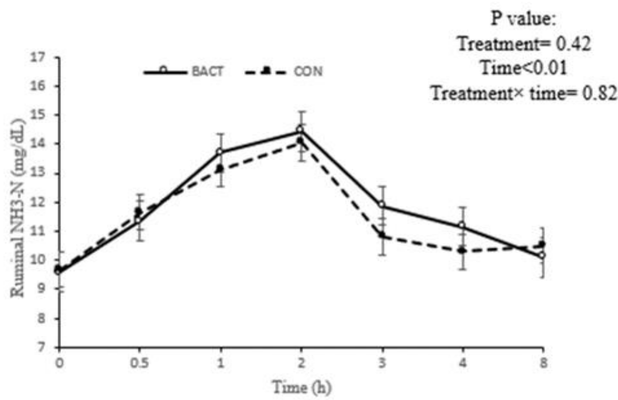

Figure 3. *In vivo* effect of inoculation of an antibiotic resistant bacterium (*Lactococcus lactis*, BACT) on ruminal N-NH_3 concentration during 8 h after the morning feeding in rumen- fistulated lactating Holstein cows: Control (CON) *vs.* BACT (cfu= 1×10^{11}, BACT).

Ruminal nutrient disappearance

Referring to Table 5, the administration of BACT in the rumen improved the apparent digestibility of DM (74.28 vs. 71.55), CP (77.99 vs. 75.45), NDF (64.26 vs. 60.24), ADF (62.68 vs. 56.03) and EE (88.77 vs. 85.33), whereas, OM digestibility was not affected (P>0.05).

Ruminal bacterial abundance

The abundance of ruminal cellulolytic bacteria, *R. flavefaciens* (11.86 vs. 9.02) and lactate utilizer *M. elsdenii* (29.07 vs. 25.88) was greater (P>0.05) in samples from cows receiving the BACT compared to the CON group (Table 6). In contrast, the abundance of *S. bovis* remained unchanged between the BACT and CON groups.

Table 3. *In vivo* effect of inoculation of an antibiotic resistant bacterium (*Lactococcus lactis*, BACT) on ruminal volatile fatty acid (VFA) concentration in lactating Holstein cows.

	Treatments			
Item	CON	BACT	SEM*	*P*-value
Total VFA (mM)	100.22	109.81	0.71	0.01
Individual VFA (mmol/100 mol of VFA)				
Acetate	64.45	76.17	3.97	0.04
Propionate	19.25	22.44	2.33	0.09
Butyrate	11.78	14.98	1.88	0.11
Valerate	1.56	1.96	0.18	0.06
Iso-butyrate	1.12	1.37	0.11	0.06
Iso-valerate	1.64	2.02	0.27	0.19

CON: basal diet only; BACT: inoculated transgenic *Lactococcus lactis* bacteria (cfu= 1×10^{11})
* SEM: standard error of the mean

Table 4. *In vivo* effect of inoculation of an antibiotic resistant bacterium (*Lactococcus lactis*, BACT) on dry matter intake, milk yield and milk composition in lactating Holstein cows.

	Treatments			
Item	CON	BACT	SEM*	*P*-value
Dry matter intake (kg/d)	17.61	18.16	1.32	0.61
Milk yield (kg/d)	28.73	29.31	0.68	0.33
3.5% FCM[1] (kg/d)	28.72	30.92	0.56	0.04
Milk composition				
Milk fat (%)	3.54	3.80	0.13	0.03
Milk protein (%)	3.26	3.14	0.74	0.24
Lactose (%)	4.52	4.55	0.05	0.43
Milk fat yield (kg/d)	1.01	1.13	0.01	0.01
Milk protein yield (kg/d)	0.93	0.92	0.05	0.68
Milk Lactose yield (kg/d)	1.29	1.33	0.04	0.41
Milk urea nitrogen (mg/dL)	14.23	13.52	0.37	0.55

[1] 3.5% FCM= 0.43 (kg of milk production/d) + 16.216 (kg of fat/d)
CON: basal diet only, BACT: inoculated transgenic *Lactococcus lactis* bacteria (cfu= 1×10^{11})

Table 5. *In vivo* effect of inoculation of an antibiotic resistant bacterium (*Lactococcus lactis*, BACT) on total tract apparent digestibility of nutrients in lactating Holstein cows.

	Treatments			
Nutrients	CON	BACT	SEM*	*P*-value
Dry matter	71.55	74.28	0.52	0.01
Organic matter	74.58	75.07	0.67	0.67
Crude protein	75.45	77.99	0.56	0.01
Neutral detergent fiber	60.24	64.26	0.52	0.01
Acid detergent fiber	56.03	62.68	0.74	0.01
Ether extract	85.33	88.77	0.42	0.02

CON: basal diet only, BACT: inoculated transgenic *Lactococcus lactis* bacteria (cfu= 1×10^{11})
* SEM: standard error of the mean

Table 6. *In vivo* effect of inoculation of an antibiotic resistant bacterium (*Lactococcus lactis*, BACT) on the abundance of ruminal bacteria (after 8 h) in lactating Holstein cows.

Bacteria	Treatments		SEM*	*P*-value
	CON	BACT		
Streptococcus bovis	24.15	24.89	1.74	0.77
Megasphaera elsdenii	25.88	29.07	1.15	0.03
Ruminococcus flavefaciens	9.02	11.86	1.25	0.04
Lactococcus lactis	19.71	21.57	1.46	0.39

CON: basal diet only, BACT: inoculated transgenic *Lactococcus lactis* bacteria (cfu= 1×10^{11})
* SEM: standard error of the mean

Discussion

Acidosis occurs when the pH in the rumen drops below 5.6 (Hernández et al., 2014). In this study, the ruminal pH measurements indicated that acidosis was successfully induced during the experimental period feeding corn grain meal or whey powder (Figure 2). The impact of bacterial inoculation on ruminal pH values has been examined by Yusuf and Abdul Hamid (2013). Results from previous studies showed no negative effects of administering LAB on rumen pH, and some even reported higher pH values. Chiquette et al. (2015) investigated the effects of supplying LAB to dairy cows challenged with sub-acute ruminal acidosis (SARA) and found positive effects on ruminal pH. Similar findings were reported by Gotto et al. (2016) in non-lactating cattle, where the 24 h mean ruminal pH was higher in the LAB-treated groups compared to the control group during the SARA challenge. In another study, LAB, including *Lactobacillus acidophilus* and *Enterococcus faecium*, increased the ruminal pH over time in steers fed a finishing diet (Kenney et al., 2015). Raeth-Knight et al. (2007) reported that the use of microbial supplements; *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* in mid-lactation Holstein dairy cows fed a 41% concentrate-based diet, did not impact on the ruminal pH. This suggests that the mechanism of action of these inoculants is more likely to influence the predominance of certain ruminal microbes rather than directly by LAB fermenting substrates (Chiquette et al., 2012). Furthermore, bacterial probiotics may help prevent a decline in ruminal pH by enhancing the lactic acid consumption by specific microbes (Chiquette et al., 2008). The current results indicated that BACT did not impact on rumen NH_3-N concentration. It appears that ruminal NH_3-N concentration is primarily influenced by dietary protein content and degradability in the rumen. However, Monteiro et al. (2020) studied the effects of DFM containing *Lactiplantibacillus plantarum* and *Lactobacillus acidophilus* in high-producing dairy cows and reported a reduction in ruminal NH_3-N concentration compared to those not supplemented with DFM.

Ruminal VFAs serve as the primary energy source for ruminants, providing up to 80% of their energy requirements (Gonzalez-Aguilar et al., 2016). The content and composition of VFAs are important physiological indices that play a crucial role in reflecting rumen digestion and metabolism. Probiotics can promote the growth of rumen epithelial cells, thereby enhancing nutrient uptake capacity by improving VFA production (Nalla et al., 2022). The present study found that rumen inoculation with BACT significantly increased the total VFA concentration and the molar proportion of acetate. Additionally, the concentrations of propionate, valerate, and isobutyrate tended to increase ($0.05<P<0.1$) in BACT group. We hypothesized that BACT would indirectly increase ruminal propionate levels by producing lactic acid, which would then serve as a substrate for propionate-producing bacteria. Results from previous studies indicated that feeding LAB alone or in combination with *L. acidophilus* and *P. freudenreichii* did not affect rumen propionate levels, either *in vitro* (Meissner et al., 2014) or *in vivo* (Raeth-Knight et al., 2007; Kenney et al., 2015). Similarly, Cao et al. (2010) found that the *in vitro* fermentation of a total mixed ration inoculated with *lactobacillus plantarum* (*L. plantarum*) did not influence the total VFA concentration or VFA profile. In another study, the authors reported that the addition of LAB (a combination of *L. plantarum* and *P. pentosaceus*) resulted in an increase in propionic acid levels in the rumen (Chen et al., 2016). Rabelo et al. (2018) also observed that the incorporation of *L. plantarum* and *Enterococcus faecalis* in animals fed corn stover silage increased propionate levels and reduced acetate/propionate ratios in an *in vitro* study. A previous study reported that providing calves with a multi-strain probiotic feed additive containing *Lactobacillus casei* PKM B/00103, *Lactobacillus salivarius* PKM B/00102, and *Lactobacillus sakei* PKM B/00101, resulted in enhanced ruminal fermentation and increased concentrations of total VFA, propionate, and butyrate (Stefanska et al., 2021). The VFA profile in the rumen is of particular interest because any alterations in VFA may indicate changes in ruminal fermentation patterns. Probiotics have been shown to enhance growth and/or cellulolytic activity in rumen bacteria, as well as preventing ruminal acidosis by maintaining a balance in VFA ratios within the rumen (Arcos-Garcia et al., 2000). This was confirmed in our study, where the population of *R. flavefaciens*, being a predominant cellulolytic and bacterium that produces acetate, was increased significantly. Dry matter intake did not show any significant differences between the experimental treatments, which may explain the lack of an increase in milk production observed in our experiment. Previous studies have indicated that probiotics can improve milk production in early-lactation cows (Nocek and Kautz, 2006). In contrast, some studies reported that lactic acid-

producing bacteria significantly increased milk production, milk protein percentage, and non-fat DM content in dairy cattle (Chen et al., 2013). The observed increase in the proportion of milk fat within the BACT group may be attributed to improved fiber digestion in the rumen, leading to a higher ruminal acetate-to-propionate ratio. According to Seymour et al. (2005), there is a positive correlation between the concentration of acetate in the rumen and the content of milk fat; thus, increased availability of acetate could facilitate greater milk fat production. The other milk components were not affected by bacterial inoculation, which is consistent with the study conducted by Tesfaye and Hailu (2019), where supplementing 10^9 cfu of *Lactobacillus acidophilus* per cow did not influence milk composition. The unchanged concentration of MUN in the BACT group in the current study could be explained by the unaffected utilization of NH_3-N in the rumen following the administration of bacterial inoculation.

Lactic acid bacteria are known to enhance nutrient disappearance (Mudgal and Baghel, 2010). Weinberg et al. (2007) reported that certain LAB inoculants, when applied during the ensiling process or directly inoculated into rumen fluid, could potentially increase the digestibility of DM and NDF. This increase in nutrient digestibility may be attributed to the action of hydrolytic enzymes produced by the bacteria. Kim et al. (2017) found that when *L. plantarum* is used as a silage inoculant, it releases enzymes such as cellulase, xylanase, chitinase and esterase. The addition of *Bacillus licheniformis* to the diet of lactating cows increased the disappearance of NDF (Qiao et al., 2010). Similarly, Boyd et al. (2011) discovered that lactating cows fed a diet supplemented with *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* exhibited increased NDF and protein disappearance. Moreover, Daniel et al. (2018) found that DM digestion increased in dairy cows fed corn silage inoculated with a mixture of *L. lactis*, *L. plantarum*, and *E. faecium*. In the study by Nayel et al. (2019), feeding *Lactococcus lactis* as a probiotic to calves, enhanced the disappearance of CP, CF, and EE. However, other studies reported varying effects on silage disappearance due to LAB inoculation. Ellis et al. (2015) noted similar NDF disappearance between dairy cows fed ryegrass silage inoculated with *L. plantarum*, *L. buchneri*, *L. lactis*, and the control group. Similarly, the total-tract apparent digestibility of DM, OM and, NDF did not change when dairy cows were directly fed *Propionibacterium*, *L. plantarum* and, *Lacticaseibacillus rhamnosus* (Philippeau et al., 2017).

Administration of LAB is believed to help the ruminal microbiota adapt to the presence of lactic acid (Ghorbani et al., 2002) and enhance the ability of rumen bacteria to metabolize lactic acid (Qadis et al., 2014). Furthermore, the transgenic bacterium, used in our study, which expresses AMP (cLF), has demonstrated antibacterial activity against several significant foodborne pathogens (Tanhaieian et al., 2020). According to Tang et al. (2009), supplementation with a fusion peptide of bovine lactoferricin-lactoferrampin reduced the concentration of *E. coli* in the ileum, cecum, and colon, while increasing the levels of *Lactobacillus* and *Bifidobacteria*. Kawai et al. (2003) investigated the effects of lactoferrin hydrolysate in cows suffering from subclinical mastitis caused by bacteria, including *E. coli* and *staphylococci*, and reported a significant reduction in bacterial counts in the mammary tissue. Since some LAB can survive and proliferate in ruminal fluid (Weinberg et al., 2004), and the bacteria used in our experiment have been tested and shown to survive in ruminal fluid (Yahfoufi et al., 2024), we anticipated an increase in the proportion of *L. lactis* in the BACT group. However, the proportion of *L. lactis* was not significantly affected by inoculation. The stable ruminal pH observed in BACT may indicate a higher proliferation of *R. flavefaciens* in the rumen. Furthermore, the increase in the abundance of *R. flavefaciens* as major cellulolytic bacteria in the rumen, is attributed to enhanced degradability of DM or fermentation metabolites produced by LAB (Astuti et al., 2022). Similar findings were reported by Oskoueian et al. (2021). The increase in fibrolytic bacteria also serves as an evidence that supplementation with LAB as probiotics did not negatively impact ruminal fermentation (Astuti et al., 2022). The lactic acid-producing bacteria used in our study may influence ruminal lactic acid concentration by enhancing lactate consumption, thereby stabilizing ruminal pH through a reduction in lactate concentration via increased activity of lactate-consuming bacteria (Susanto et al., 2023). This is supported by our observation of a higher abundance of *M. elsdenii* in the BACT treatment. Additionally, these bacteria can inhibit the activity of lactate-producing bacteria, particularly *S. bovis* (Chaucheyras et al., 1995). However, our results suggested that the products formed by BACT may be specific to certain microorganisms. The abundance of *S. bovis* was not influenced by the use of BACT, which may be attributed to its resistance to the BACT employed in the study (McAllister et al., 2011). Although probiotic products typically contain a low number of viable microorganisms, the optimal composition and dosage of these probiotics remain unclear (Fasoli et al., 2003). Such products may enhance the effects of microorganisms predominantly present in the rumen (Weinberg et al., 2004). However, antagonistic interactions and potential inhibitory effects between ruminal bacteria and inoculated probiotic strains have also been documented (Chiquette et al., 2012). While the mechanism of action of probiotics in the rumen is not fully understood, the administration of LAB probiotics is believed to assist the ruminal microflora in adapting to the presence of lactic acid (Ghorbani et al., 2002) and to prevent lactate accumulation in the rumen.

Conclusion

It was concluded that the inoculation of BACT exerted no negative effects on pH and N-NH_3 concentration, while an increase in milk fat content was accompanied by an improvement in total tract apparent nutrient digestibility.

The BACT also altered ruminal VFA concentration, resulting in a higher production of acetate. The abundance of lactate-utilizing bacteria (*M. elsdenii*) and cellulolytic bacteria (*R. flavefaciens*) increased by BACT inoculation. However, further feeding studies on a farm scale need to be conducted with more animals to confirm the effects of these bacteria on animal health and productive responses, particularly when challenged with a type of acidosis.

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