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In vitro addition of antibiotic resistant bacteria (*Lactococcus lactis*) modulates ruminal nutrient disappearance kinetics of diets varying in lactose/starch ratios

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Abstract *In vitro* ruminal culture was carried out to assess the effect of the concentration of an antibiotic resistant transgenic bacterium (*Lactococcus lactis, L. l*actis) along with different dietary lactose/starch ratios on first order kinetic disappearance of nutrients using an exponential model. Dietary treatments were designed to include lactose (L), using whey powder which was substituted of corn grain, as zero ($L_{0.0}$), 36 (L_{36}) and 72 (L_{72}) g/kg dry matter (DM), with different *L. l*actis count (CFU/mL) as 0.0 ($Bact_{0.0}$), 1.25×10^8 ($Bact_{1.25}$) and 2.5×10^8 ($Bact_{2.50}$) in a 3×3 factorial arrangement. The DM and CP disappearance was significantly influenced by the dietary inclusion of *L. lactis*. Constant rate of crude protein (CP) disappearance significantly decreased in both L_{36} and L_{72} using bacteria as $Bact_{2.50}$. $Bact_{2.50}$ enhanced the constant rate of neutral detergent fiber (NDF) disappearance of the diets. DM disappearance was significantly increased in lactose containing diets, while crude protein disappearance was significantly enhanced in L_{36} only. NDF disappearance decreased significantly in L_{36} and L_{72} treatments, and the indigestible fraction of NDF was significantly higher for L_{72} compared with $L_{0.0}$ or L_{36}. NFC digestible fraction was significantly improved in L_{36} and L_{72}.There was significant diet× bacteria interaction in most of the studied parameters. Ruminal *L. lactis* abundance measured using RT-PCR altered in all diets, and the highest values were recorded in L_{72} diet. Regarding the dietary inclusion of lactose, some alterations were detected, while the effect of the bacteria on nutrient disappearance was consider and related to the dietary lactose/starch ratios.

Keywords: antimicrobial peptide, disappearance kinetics, *Lactococcus lactis*, lactose

Introduction

The diets of dairy cattle often include increased non-fiber carbohydrates (NFCs), such as starch, to maximize milk production (Gao and Oba, 2016). However, excess starch intake causes metabolic disorders such as subacute ruminal acidosis that is associated with low rumen pH, milk fat depression (Kleen et al., 2003), diminished dry matter intake (DMI; Aschenbach et al., 2011) and decreased neutral detergent fiber (NDF) digestibility (Krause and Oetzel, 2005). The composition of rumen microbiota can also be altered including an increase in amylolytic bacteria and a decrease in cellulolytic bacteria (Fernando et al., 2010). Sugars can partially substitute starch and often increase fiber digestion (Vallimont et al., 2004), DMI (Broderick

et al., 2008) and milk fat yield (Baurhoo and Mustafa, 2014) without adverse effect on pH (Penner and Oba, 2009). Lactose, a major carbohydrate in whey powder (Oba, 2011), increased the DMI (Charbonneau et al., 2006) and apparent total tract digestibility of fat with a tendency to increase the dry matter (DM) digestibility (Chibisa et al., 2015). From the economical point of view, replacing grains with sugar can lower the feeding costs (Gao and Oba, 2016).

Antibiotics, used as growth promoters, have been banned due to several disadvantages, including their resistance in both animals and humans (Benchaar et al., 2006). This has led to the search for alternatives, such as antimicrobial peptides from bacteria (Adeniyi et al., 2015). Lactic acid-producing bacteria (LAB) can facilitate growth of rumen microorganisms adapted to acidic environments and are often used in preservation of ensiled feed (Guo et al., 2022). Yoon and Stern (1995) reported that feeding *Lactobacillus acidophilus* bacteria to calves, steers and dairy cows fed a high-concentrate diet resulted in increased daily weight gain, milk production and improved feed efficiency. Similar results were obtained in a study of Elam et al. (2003) when *L.acidophilus* or *Propionibacterium freudenreichii* were fed to feedlot cattle. It has been reported that feeding *Megasphaera elsdenii* to cattle prevented lactate accumulation in the rumen and reduced the occurrence of acidosis (Kung, 2006). *Lactococcus lactis*, a gram-positive bacteria found in dairy products (Birollo et al., 2000), produces lactic acid from glucose fermentation, where low pH inhibits the growth of pathogenic bacteria in the intestine (Dougherty et al., 2002). A heterodimeric peptide “cLF chimera”, designed to mimic two antimicrobial domains, Lactoferricin and Lactoferrampin (camel milk antimicrobial peptides), has been cloned and expressed in *L. lactis*. *In vitro*, it exerted inhibitory effects against the gram negative (*E. coli*, *Salmonella enteritis*, *Pseudomonas aeuruginosa*) and positive (*Staphylococcus aureus*) species (Tanhaeian et al., 2020). Tang et al. (2009) showed that bovine lactoferricin-lactoferrampin in piglets decreased *E-coli* count while increased lacto-bacilli and bifidobacteria in ileum, cecum and colon, and increased villus height of jejunum and ileum. This study aimed to determine the effect of adding a transgenic bacterium (*L. lactis*), expressing cLF chimera, on *in vitro* ruminal disappearance kinetics of nutrients from diets containing different sources of NFC (corn rich in starch vs whey rich in lactose), and on *L. lactis* abundance measured by real-time PCR (RT-PCR).

Materials and methods

Test of bacterial survivability in ruminal fluid

To investigate bacterial survivability in the ruminal fluid, three consecutive tests were performed using spread plate procedure (Devika et al., 2019). In the first assay, distilled water was substituted with the ruminal fluid (RF) and used as the solvent in the culture medium (M17). In the second test, buffered ruminal fluid (BRF) was used in the medium, and in the third test, bacterial strains were grown overnight in M17 broth and then culture volume was mixed with RF or BRF at different ratios (1:1, 0.4:0.6, 0.25:0.75). The cultures were incubated at 38.6°C for 16-18 h and then streaked onto M17 plates. In these tests, the volume of starting bacteria inoculant was increased to 10µL/mL, 20µL/mL and 60µL/ mL. The plates were then observed for growth.

Lactococcus lactis for inoculant preparation and experimental diets

Based on the results of the preliminary tests, a starting volume of bacterial inoculant was selected for overnight culture at 38.6°C using culture broth method (Bonnet et al., 2020). The treatment factors were source of energy (starch versus lactose) and *L. lactis* inoculation. The treatments, applied in a 3×3 factorial arrangement, were starch at 25% of dietary DM ($L_{0.0}$); whey powder containing 72% lactose and substituting 5% of starch contributing to 36 g/kg DM lactose (L_{36}); and whey substituting 10% of starch with 72g/kg DM lactose (L_{72}). One of three levels of the bacteria as 0.0 (bact0.0), 1.25×10^8 (bact1.25) or 2.5×10^8 (bact2.50) CFU/mL was inoculated. Both sources of NFC were included in a diet with similar ingredients (Table 1).

In vitro batch rumen incubation

The RF was obtained before the morning feeding from 3 rumen fistulated Holstein dairy cows fed a total mixed ration (TMR). The forage portion contained alfalfa hay and corn silage as 250 and 200 g/kg DM, respectively, and the concentrate consisted of barley grain, wheat bran, soybean meal, cottonseed meal, common salt, sodium bicarbonate, calcium carbonate and mineral and vitamin supplements as 240, 141, 55, 87, 5, 7, 6 and 9 g/kg DM, respectively. The RF was strained through 4-layer cheesecloth, then centrifuged at 6,000 rpm for 5 min. The anaerobic cultural technique was that described by Dehority (1969) and the fermentation medium was prepared as suggested by Arroquy et al. (2005), where it was composed of 400 mL cell-free RF, cellobiose (0.05 g), K_2HPO_4(0.45 g), NaCl (0.9 g), $(NH_4)_2SO_4$ (0.9 g), $MgSO_4.7H_2O$ (0.09 g), $CaCl_2$ (0.09 g), Resazurin (0.01 g), $NaHCO_3$ (4 g), and cysteine-HCl (0.5 g) per liter of the medium, and autoclaved at 120°C for 20 min. A sample (45 mL) of the fermentation medium was transferred into a 100 mL bottle containing 0.5 g of the experimental diet. Then, each bottle was inoculated with 5 mL of the strained rumen liquid and bacteria and incubated for 48 h at 38.6°C. Each treatment was replicated three times and all operations were carried out under a flux of CO_2. After 8-h incubation, 5 mL of each sample were collected and preserved at -80°C for *Lactococcus lactis*-community composition analyses.

Table 1. Ingredients and nutrient composition of dietary treatments

	Experimental diets†		
	$L_{0.0}$	L_{36}	L_{72}
Ingredients (g/kg DM)	175	175	175
Alfalfa hay	175	175	175
Corn grain	175	125	75
Barely grain	175	175	175
Soybean meal	150	150	150
Wheat bran	150	150	150
Whey	-	50	100
Nutrient composition (% DM)			
Crude protein	18.20	17.85	17.88
Neutral detergent fiber	33.20	32.26	29.33
Ether extract	7.86	7.26	8.00
Non-fibrous carbohydrate	35.14	36.43	38.99
Ash	5.60	6.20	5.80
Starch	25.00	21.40	17.80
Lactose	0	3.60	7.20

†) Corn grain-based diet, no added whey powder ($L_{0.0}$), was substituted with whey powder providing lactose at 36 (L_{36}) and 72 (L_{72}) g/kg DM.

Chemical analysis

The diets were analyzed for DM, crude protein (CP), ash and ether extract (EE) as described by AOAC (1995), and NDF was analyzed based on Van Soest et al. (1991). The NFC content of the experimental diets was calculated as follows:
NFC (g/kg DM) = 1000 - [NDF (g/kg DM) + CP (g/kg DM) + EE (g/kg DM) + ash (g/kg DM)].

DNA extraction

The RF samples were thawed at room temperature and a total of 500 micro liters of each sample was centrifuged at 10000 rpm for 3 min to collect the sediment. Subsequently, total DNA was extracted from the sediment using spin column method (Boom et al., 1990) with the FavoPrep™ Tissue Genomic DNA Extraction Mini Kit. The DNA concentration and purity were determined using a Nanodrop spectrophotometer. The DNA quality was also evaluated by Nanodrop and applied to 1% (w/v) agarose gel electrophoresis. All DNA samples were stored at -80°C. A PCR was performed with the corresponding forward and reverse primers of *L. lactis*. Samples were run in triplicate and the cycling conditions were: 4 min initial denaturation at 95°C; 35 cycles of denaturation at 95°C (30 s); annealing at 62°C (30 s), elongation at 72°C (20 s); and final extension at 72°C for 10 min. The PCR products were separated by 1% gel electrophoresis.

Quantitative real-time PCR analysis

Quantitative RT-PCR was performed in 96-well optical plates CFX96 RT-PCR detection System (Bio-Rad, USA) to investigate the abundance of previously isolated *L. lactis* (Kubista et al., 2006). The reaction was performed in total of 15-µL PCR mixture using 7.5 µL of SYBR green PCR master mix, 1µL of forward primer (10µmol/L), 1µL of reverse primer (10µmol/L) (targeting the 16S rRNA gene of the selected bacteria) 2µL of DNA, and 3.5 µL of PCR grade water. Amplification was consisted of denaturation of 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 62°C for annealing, 20 s at 72°C for extension and 10 min for elongation at 72°C. Each reaction mixture was run in triplicate and negative controls were loaded to screen for possible contamination and dimer formation. The C_T (number of fold difference) was determined during the exponential phase of amplification, and mean Ct of triplicates of each sample was used for calculations.

Calculation and statistical analysis

The DM after 48 h of incubation was calculated as the difference between DM content of the substrate before incubation and its undegradable DM after incubation. First order parameters of DM, CP, NDF and NFC disappearance were determined using first order exponential model. The model was: $D_{(t)} = D_{(i)} . e^{(-K_d . time)} + I$, where: $D_{(t)}$= potentially digestible fraction, $D_{(i)}$= potentially digestible residues, K_d= fractional rate constant of digestion (/h), I= indigestible fraction. Data were analyzed as a balanced completely randomized design with a factorial arrangement using the MIXED procedure (SAS, 2004) according to a statistical model of $Y_{ij} = \mu + A_i + B_j + (AB)_{ij} + e_{ij}$, where Y_{ij} is the measured value, µ is the overall mean, A_i is main effect of source of carbohydrate (i= 1 to 3), B_j is the main effect of bacteria count (j= 1 to 3), $(AB)_{ij}$ is the interaction between A and B and e_{ij} is the residual error. Differences among the least square means were tested using the Tukey's multiple comparison test. Significance was declared at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

Results

Lactococcus lactis survivability and abundance

In the survivability assay, no bacterial growth was observed when liquid cultures of bacteria were spread in

agar plates where distilled water was replaced with either RF or BRF as a solvent of M17 culture medium (Figure 1). When bacteria were inoculated in their selected growth medium and then mixed with either RF or BRF, growth was obtained over the agar plates. As the ratio of M17 culture medium to BRF (1:1, 0.6:0.4, 0.75:0.25), and the initial volume of inoculant (10µL to 60µL) were greater, more colonies were detected (Figures 2, 3 and 4).

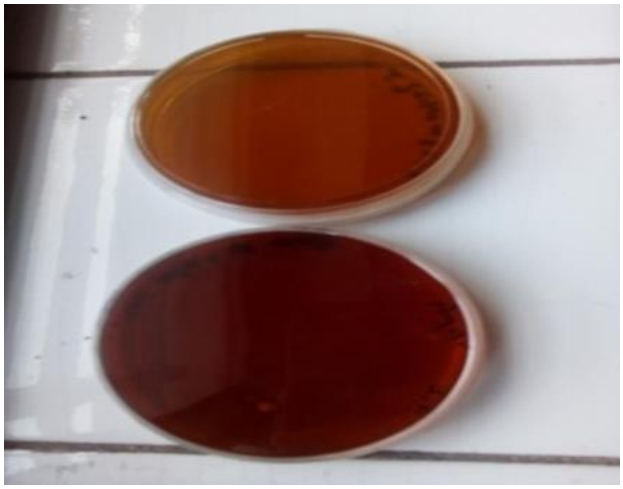

Figure 1. The result of spreading liquid cultures of bacteria (Transgenic *L. lactis*) where distilled water was used as the solvent of M17 culture medium in lieu of either ruminal fluid or buffered ruminal fluid.

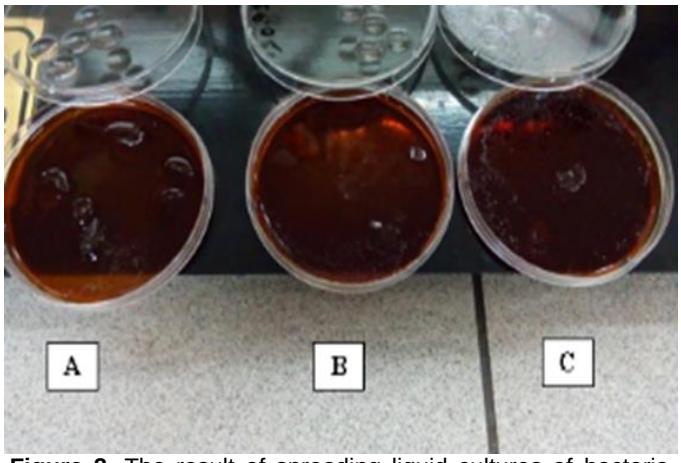

Figure 2. The result of spreading liquid cultures of bacteria (transgenic *L. lactis*) mixed with buffered ruminal fluid (BRF) (10µL initial inoculated volume) on agar media plates with different M17 culture medium: BRF volume ratio (1:1(A), 0.6:0.4(B),0.75:0.25(C)).

The ruminal microbial abundance for *L. lactis*, shown in the results of RT-PCR technique, indicated that it was sensitive to starch substitution by lactose (Table 2). Lactose inclusion at low dose caused significant reduction in *L. lactis*. However, increasing lactose inclusion increased *L. lactis* abundance. Transgenic *L. lactis* inclusion did not exert an effect on *L. lactis* abundance. However, the interaction effect of diet and bacteria was significant.

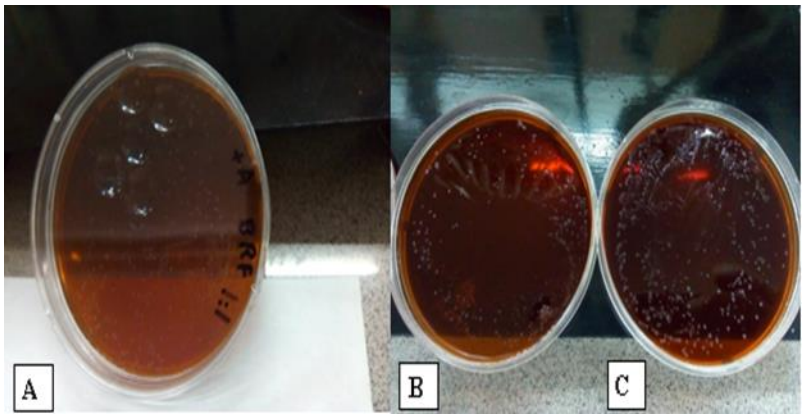

Figure 3. The result of spreading liquid cultures of bacteria (transgenic *L. lactis*) mixed with buffered ruminal fluid (BRF) (20µL initial inoculated volume on agar media plates with different M17 culture medium: BRF volume ratio (1:1(A), 0.6:0.4(B), 0.75:0.25(C)).

Ruminal nutrient disappearance

Data on the ruminal disappearance of DM, CP, NDF and NFC after 48 h incubation are shown in Table 3. Substituting starch with lactose exerted a significant positive effect on DM disappearance in both L_{36} and L_{72} ($P<0.01$). Regarding bacterial effect in L_{72} treatment, $Bact_{2.50}$ resulted in a numerical decrease in DM disappearance compared with $L_{0.0}$ treatment after 48 h incubation. However, low inclusion level of bacteria ($Bact_{1.25}$) significantly increased DM disappearance in L_{36} treatment. There was a significant diet×bacteria effect after 48 h incubation. With regard to CP disappearance, a significant increase was observed in L_{36} treatment after 48 h incubation, moreover bacterial inclusion exerted a positive effect on CP disappearance in L_{72} treatment. A significant diet×bacteria effect was recorded. The NDF disappearance decreased significantly in both L_{36} and L_{72} treatments compared to $L_{0.0}$ treatment. Bacterial inclusion did not exert any

significant effect on NDF disappearance, but numerically increased the NDF disappearance in L_{36} treatment; however, there was a significant diet×bacteria effect at 48 h incubation. Considering the NFC disappearance, no significant effect was recorded by substituting starch with lactose at both levels. Regarding the bacterial effect, no significant effect (P>0.05) was shown in dietary treatments. In general, the effect of dietary levels of *L. lactis* on nutrients disappearance was inconsistent; however, nutrient disappearance was affected by the interaction of lactose inclusion and bacterial inoculation.

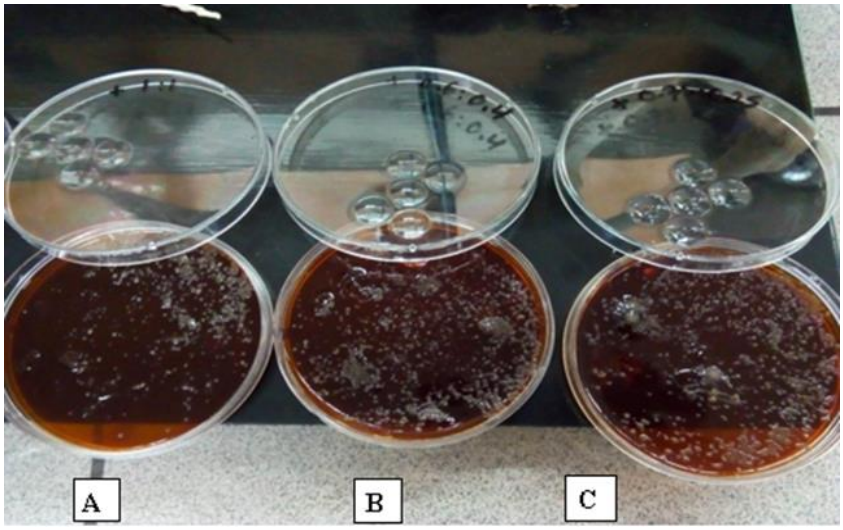

Figure 4. The result of spreading liquid cultures of bacteria (transgenic *L. lactis*) mixed with buffered ruminal fluid (BRF) (60µL initial inoculated volume) on agar media plates with different M17 culture medium: BRF volume ratio (1:1(A), 0.6:0.4 (B), 0.75:0.25 (C)).

Table 2. Effect of the level of transgenic bacteria *L. lactis* along with different dietary lactose/starch ratios on the abundance of *L. lactis* after 8 h of *in vitro* incubation

	Experimental diets												
	$L_{0.0}$[1]			L_{36}[2]			L_{72}[3]				*P*-value		
Microorganism	$Bact_{0.0}$[5]	$Bact_{1.25}$[6]	$Bact_{2.50}$[7]	$Bact_{0.0}$	$Bact_{1.25}$	$Bact_{2.50}$	$Bact_{0.0}$	$Bact_{1.25}$	$Bact_{2.50}$	SEM*	Diet	Bacteria[4]	Diet×Bacteria[8]
Lactococcus lactis	25.18^{b}	25.04^{b}	25.54^{ab}	23.86^{cd}	23.54^{d}	24.44^{c}	26.58^{a}	26^{a}	26.13^{a}	0.3	<0.01	0.18	<0.01

* SEM: standard error of the mean, [1] Corn grain-based diet (no added lactose), [2] Corn grain substituted with whey powder providing lactose at 36g/kg DM, [3] Corn grain substituted with, whey powder providing lactose at 72 g/kg DM, [4] Transgenic *Lactococcus lactis,* [5] No added transgenic *Lactococcus lactis,* [6] Transgenic *Lactococcus lactis* inoculated at 1.25×10^{8} CFU, [7] Transgenic *Lactococcus lactis* inoculated at 2.50×10^{8} CFU, [8] Interaction of diet and bacterial inclusion level
[a, b:] Within rows, mean values with common superscript (s) are not different (P>0.05; Tukey's test).

In vitro first order kinetics of nutrient (DM, CP, NDF and NFC) disappearance are shown in Table 4. Potentially digestible fraction of DM ranged from 0.47 to 0.63. The addition of lactose resulted in an improvement in potentially digestible DM fraction at $Bact_{0.0}$ and $Bact_{1.25}$ inclusion levels. The fractional rate constant of DM disappearance ranged from 0.14 to 0.33 (/h), and the highest values of fractional rate constant of DM disappearance values were recorded in L_{36}. Indigestible fraction of DM was significantly lower for treatments containing lactose (with no or low-level bacterial inoculation) compared with $L_{0.0}$ treatment. Bacterial inclusion ($Bact_{2.50}$) decreased the potentially digestible fraction of DM, in L_{36} treatment, where both $Bact_{1.25}$ and $Bact_{2.50}$ decreased it in L_{72} treatment. Bacterial inclusion caused a significant decrease in fractional rate constant of DM disappearance in L_{36} treatment. The potentially digestible fraction of CP was the highest in L_{36} diet, and the $bact_{1.25}$ enhanced significantly the digestible fraction of CP in L_{36} treatment. The constant rate of CP disappearance significantly decreased in both L_{36} and L_{72} using bacteria as $Bact_{2.50}$ (P<0.05). The NDF potentially digestible fraction was similar between $L_{0.0}$ and L_{36} diet; however, it was negatively affected by the higher lactose inclusion level (L_{72}). The indigestible fraction of NDF was significantly increased in L_{72} in comparison with $L_{0.0}$ and L_{36} treatments. With regard to the bacteria, *L. lactis* inclusion exerted a negative effect on the NDF digestible fraction in L_{72} where the reduction was significant at both inoculation levels; however, only low level of bacteria ($Bact_{1.25}$) decreased NDF digestible fraction in $L_{0.0}$ and L_{36} treatments. The fractional rate constant of NDF disappearance was influenced positively by bacterial inclusion at higher level ($Bact_{2.50}$) in all treatments compared with $Bact_{0.0}$. The NFC digestible fraction was increased by lactose significantly in L_{36} and L_{72} treatments. The fractional rate constant of NFC disappearance was not significant among the

different treatments. Bacterial inclusion ($Bact_{2.50}$) exerted a positive effect on the NFC digestible fraction in the L_{36} diet; however, in L_{72}, both bacterial levels decreased the potentially digestible fraction of NFC. Except for the fractional rate constant of DM and NFC disappearance, there was a significant diet×bacteria effect on other reported parameters.

Table 3. Effect of the level of transgenic bacteria *L. lactis* along with different dietary lactose/starch ratios on nutrient disappearance after 48 h of *in vitro* incubation

	Experimental diets												
	$L_{0.0}$[1]			L_{36}[2]			L_{72}[3]				*P*-value		
Nutrients	$Bact_{0.0}$[5]	$Bact_{1.25}$[6]	$Bact_{2.50}$[7]	$Bact_{0.0}$	$Bact_{1.25}$	$Bact_{2.50}$	$Bact_{0.0}$	$Bact_{1.25}$	$Bact_{2.50}$	SEM*	Diet	Bact[4]	Diet×Bact[8]
dry matter	0.5^{c}	0.5^{c}	0.51^{c}	0.6^{b}	0.66^{a}	0.59^{b}	0.65^{a}	0.64^{a}	0.62^{ab}	0.013	<0.01	0.03	0.02
Crude protein	0.66^{b}	0.65^{b}	0.64^{b}	0.73^{a}	0.72^{a}	0.71^{a}	0.6^{c}	0.66^{b}	0.64^{b}	0.018	<0.01	0.037	0.02
Neutral detergent fiber	0.91^{a}	0.92^{a}	0.93^{a}	0.81^{bc}	0.84^{b}	0.86^{b}	0.83^{b}	0.81^{bc}	0.84^{b}	0.017	<0.01	0.12	<0.01
Non-fibrous carbohydrate	0.91	0.93	0.89	0.92	0.93	0.96	0.95	0.93	0.94	0.04	0.13	0.76	0.07

* SEM: standard error of the means., [1] Corn grain-based diet (no added lactose), [2] Corn grain substituted with whey powder providing lactose at 36g/kg DM, [3] Corn grain substituted with whey powder providing lactose at 72 g/kg DM, [4] Transgenic *Lactococcus lactis,* [5] No added transgenic *Lactococcus lactis,* [6] Transgenic *Lactococcus lactis* inoculated at level 1.25 ×10^{8} CFU, [7] Transgenic *Lactococcus lactis* inoculated at level 2.50×10^{8} CFU, [8] Interaction of experimental diet and bacterial inclusion level

[a, b]: Within rows, mean values with common superscript (s) are not different (P>0.05; Tukey's test).

Table 4. Effect of the level of transgenic bacteria *L. lactis* along with different dietary lactose/starch ratios on the first order kinetics of DM, CP, NDF and NFC

	Experimental diets												
Parameters[†]	$L_{0.0}$[1]			L_{36}[2]			L_{72}[3]				*P*-value		
	$Bact_{0.0}$[5]	$Bact_{1.25}$[6]	$Bact_{2.50}$[7]	$Bact_{0.0}$	$Bact_{1.25}$	$Bact_{2.50}$	$Bact_{0.0}$	$Bact_{1.25}$	$Bact_{2.50}$	SEM*	Diet	Bact[4]	Diet×Bact[8]
Dry matter													
D	0.47^{c}	0.47^{c}	0.48^{c}	0.54^{b}	0.53^{b}	0.49^{c}	0.63^{a}	0.54^{b}	0.47^{c}	0.013	<0.01	0.02	<0.01
I	0.52^{a}	0.53^{a}	0.52^{a}	0.45^{c}	0.46^{c}	0.51^{ab}	0.36^{e}	0.44^{cd}	0.53^{a}	0.008	<0.01	<0.01	<0.01
K_d	0.16^{c}	0.15^{c}	0.14^{cd}	0.33^{a}	0.24^{b}	0.23^{b}	0.17^{c}	0.15^{c}	0.16^{c}	0.012	0.03	0.04	0.16
Crude protein													
D	0.6^{c}	0.61^{bc}	0.59^{c}	0.63^{b}	0.68^{a}	0.64^{b}	0.59^{c}	0.61^{bc}	0.6^{c}	0.011	<0.01	0.016	0.07
I	0.4^{b}	0.38^{b}	0.39^{b}	0.37^{bc}	0.32^{d}	0.35^{c}	0.43^{a}	0.39^{b}	0.37^{bc}	0.012	<0.01	<0.01	<0.01
K_d	0.21^{b}	0.2^{b}	0.2^{b}	0.23^{ab}	0.26^{a}	0.16^{c}	0.24^{a}	0.22^{b}	0.18^{bc}	0.013	0.37	0.02	0.03
Neutral detergent fiber													
D	0.65^{a}	0.6^{b}	0.68^{a}	0.67^{a}	0.62^{b}	0.65^{a}	0.36^{c}	0.25^{e}	0.31^{d}	0.025	<0.01	0.019	<0.01
I	0.33^{d}	0.37^{d}	0.3^{de}	0.33^{d}	0.37^{d}	0.34^{d}	0.63^{c}	0.75^{a}	0.69^{b}	0.022	<0.01	0.02	<0.01
K_d	0.02^{b}	0.03^{ab}	0.05^{a}	0.02^{b}	0.03^{ab}	0.06^{a}	0.03^{ab}	0.06^{a}	0.07^{a}	0.014	0.62	0.01	<0.01
Non-fibrous carbohydrate													
D	0.54^{c}	0.53^{c}	0.49^{c}	0.62^{b}	0.64^{b}	0.72^{a}	0.72^{a}	0.63^{b}	0.64^{b}	0.028	<0.01	<0.01	<0.01
I	0.45^{a}	0.47^{a}	0.5^{a}	0.38^{b}	0.35^{b}	0.27^{c}	0.27^{c}	0.36^{b}	0.35^{b}	0.027	<0.01	0.04	0.03
K_d	0.17	0.18	0.2	0.23	0.25	0.21	0.21	0.25	0.22	0.041	0.28	0.55	0.61

*SEM: standard error of the means, [†] D, potentially digestible fraction; K_d, fractional rate constant of digestion; I, indigestible fraction, [1] Corn grain-based diet (no added lactose), [2] Corn grain substituted with whey powder providing lactose at 36g/kg DM, [3] Corn grain substituted with whey powder providing lactose at 72 g/kg DM lactose, [4] Transgenic *Lactococcus lactis,* [5] No added transgenic *Lactococcus lactis,* [6] Transgenic *Lactococcus lactis* inoculated at 1.25 ×10^{8} CFU, [7] Transgenic *Lactococcus lactis* inoculated at 2.50×10^{8} CFU, [8] Interaction of experimental diet and bacterial inclusion level

[a,b]: Within rows, mean values with common superscript (s) are not different (P>0.05; Tukey's test).

Discussion

In the current study, the abundance of *L. lactis* increased at higher level of lactose inclusion (L_{72} treatment); however, the opposite results were observed in L_{36} treatment. It can be hypothesized that medium containing high level of lactose as the source of carbohydrate makes the use of this disaccharide interesting to facilitate the permanence of this microorganism in RF; however, lower lactose/starch ratio exerted negative effect on the growth of this bacteria. This could be related to the competition of starch utilizing-bacteria and to the changes in medium pH. A genetically engineered *L. lactis*, as antibiotic resistant bacteria, producing camel lactoferricin-lactoferrampin peptide impacted the nutrient disappearance kinetics when it was included in diets varying in lactose/starch ratios. Even when bacterial main effect was not noticed, interaction effect occurred between *L. lactis* inoculated level and starch/lactose

ratio. Although the effects of LAB inoculation on animal performance are not consistent, studies revealed their beneficial effects in the rumen fluid such as enhanced microbial biomass yield (Contreras-Govea et al., 2011; Basso et al., 2014) and lower methane production (Muck et al., 2007; Cao et al., 2010). As our results showed, the effect of bacterial inclusion on DM disappearance was not consistent and it was dependent on lactose/starch ratio. Previous studies have reported that when LAB were used as silage inoculants rather than as direct fed microbes, the DM disappearance was increased in both *in vitro* (Weinberg et al., 2007; Cao et al., 2011) and *in vivo* (Kung et al., 2003; Ando et al., 2006). In a study done by Daniel et al. (2018), increase in DM disappearance in dairy cows fed corn silage inoculated with a mixture of *L. lactis*, *Lactiplantibacillus plantarum* and *Enterococcus faecium* was reported. It can be hypothesized that the mechanisms of action of LAB differ depending on the administration route. The disappearance of the CP was increased upon bacterial inclusion in L_{72} diet, while it exerted no effect on the CP disappearance in other treatments. It was reported that inoculation of *L. lactis* and *Lactobacillus buchneri* in maize silage increased the CP disappearance (Nkosi et al., 2011). Considering the NDF disappearance, no effect was exerted by *L. lactis*. This result is consistent with Ellis et al. (2015) who reported that the NDF digestibility was not changed when *L. lactis* was inoculated to grass 16 h before morning feeding to dairy cows. Since LAB do not possess the enzymatic ability to hydrolyze cell-wall constituents (Rook and Hatfield, 2003), the activity of cellulolytic bacteria may not have been affected by the addition of *L. lactis*. Moreover, bacterial inclusion did not exert a significant effect on the NFC disappearance after 48 h incubation.

The effect of replacing grain starch with sugars was not consistent (McCormick et al., 2001; Penner and Oba, 2009). In our study, supplementation with lactose enhanced the DM disappearance, and higher values for DM potentially digestible fraction were recorded. The present results confirmed the findings of Sniffen et al. (1992) who reported a fermentation rate of 300%/h for sugars; a rate of disappearance of 331%/h for lactose was also reported (Weisbjerg et al., 1998). Whereas starch from corn and barely is degraded at 12 and 30%/h, respectively, some studies reported no effect of sugar addition on DM disappearance (Oelker et al., 2009; Baurhoo and Mustafa, 2014). In contrast to a previous study (Vallimont et al., 2004), partial replacement of starch from corn grain with sucrose in a TMR using dual-flow continuous-culture fermenters did not affect the apparent digestibility of DM. This increase in disappearance may be due to readily fermented carbohydrates which might have resulted in increased fermentation and microbial enzymatic activity which subsequently increased the disappearance. Moreover, microbes invest less effort to reduce sugars to smaller units compared to starch and structural carbohydrates (Golder et al., 2012). The extent of CP disappearance was significantly greater in L_{36} treatment; however, at high lactose inclusion level, no improvement was recorded. Previous *in vitro* results showed that CP disappearance was not affected by adding dried molasses at either 3 or 6% (Baurhoo and Mustafa, 2014). Another study reported a significant decrease in the apparent digestibility of the total tract CP in cows fed with 270 g/kg starch and 90 g/kg sucrose or lactose in the diets (Gao and Oba, 2016). Reports of inefficiency of CP utilization in the diets containing starch or corn may be related to lactose-utilizing microbes storing glycogen rather than fermenting it directly or competing with other microbial populations for nitrogen and other nutrients. However, we can hypothesize that lactose should be included at a lower level where high level inclusion did not show any improvement compared to $L_{0.0}$ treatment. In the current experiment, the NDF disappearance was reduced by lactose inclusion after 48 h incubation. These results are supported by Khalili and Huhtanen (1991) who showed that supplementation of sucrose (15.9%) decreased the ruminal NDF digestion. Moreover, it was reported that the rate and extent of NDF digestibility were reduced when sucrose was added to the diet (Heldt et al., 1999). Penner and Oba (2009) showed that the digestibility of NDF was not affected when corn was replaced with sucrose at either 8.4 or 4.7% levels. Also, in an *in vitro* study done by Arroquy et al. (2005), the authors reported that changing the source of supplemental NFC (glucose, maltose, cornstarch and soluble starch) did not significantly affect the NDF disappearance. This effect might be due to the sugar utilizing bacteria competing with the fiber-digesting bacteria for available N, and that the inclusion of adequate quantities of rumen degradable protein in the diet might prevent the decrease in NDF disappearance (Lee et al., 2003). It has been reported that pH did not decrease even with the high fermentation rate of sugar, thus the decrease in NDF disappearance may not be related to low pH. This was supported by Piwonka and Firkins (1993) where glucose (one of the lactose subunits) supplementation reduced the rate of NDF disappearance even if the pH was maintained above 6.2. The negative effect might also be related to lactose supplementation that caused an alteration in microbial enzymatic activity corresponding to fiber digestion such as carboxymethyl cellulase and xylanases. Hiltner and Dehority (1983) studied the effect of glucose and cellobiose on the rate of cellulose disappearance by cellulolytic bacteria (*Ruminococcus albus, Ruminococcus flavefacians and Bacteroides succinogens*) where the results showed that soluble carbohydrates shortened the lag phase and slowed the rate of cellulose disappearance. The NFC disappearance was not affected in lactose containing diets but the digestible fraction of NFC was significantly improved in L_{36} and L_{72} treatments. In a study by Poorkasegaran and Yansari (2014), in which beet pulp partially replaced barely, the NFC digestibility was reduced in lactating Holstein dairy cows.

Conclusions

In the present study, we evaluated the effect of two levels of the transgenic bacteria *L. lactis* along with different dietary lactose/starch ratios on the first order ruminal disappearance kinetics of DM, CP, NDF and NFC and on *L. lactis* abundance. The first order disappearance kinetics of nutrients was related to the inclusion percentage of lactose. The dry matter disappearance was enhanced by lactose inclusion at both levels, and that of crude protein at low level (L_{36}); however, using sugar in the experimental diets caused a negative effect on the NDF disappearance, whereas a positive effect was recorded on NFC digestible fraction by lactose. The effect of bacteria on the first order nutrient disappearance of the experimental diets was related to the ratio of starch to lactose but its effect was not consistent among diets. Generally, the interaction between the experimental diets and the bacterial inclusion was considerable and needed to be clarified in further studies.

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Conflict of interests

There is no conflict of interest.

References

AOAC, 1995. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Washington, DC. USA.

Adeniyi, B.A., Adetoye, A., Ayeni, F.A., 2015. Antibacterial activities of lactic acid bacteria isolated from cow faeces against potential enteric pathogens. *African Health Sciences* 15, 888-895.

Ando, S., Ishida, M., Oshio, S., Tanaka, O., 2006. Effects of isolated and commercial lactic acid bacteria on the silage quality, digestibility, voluntary intake and ruminal fluid characteristics. *Asian-Australasian Journal of Animal Sciences* 19, 386-389.

Arroquy, J.I., Cochran, R.C., Nagaraja, T.G., Titgemeyer, E.C., Johnson, D.E., 2005. Effect of types of non-fiber carbohydrate on *in vitro* forage fiber disappearance of low-quality grass hay. *Animal Feed Science and Technology* 120, 93-106.

Aschenbach, J.R., Penner, G.B., Stumpff, F., Gäbel, G., 2011. Ruminant Nutrition Symposium: Role of fermentation acid absorption in the regulation of ruminal pH. *Journal of Animal Science* 89, 1092-1107.

Basso, F.C., Adesogan, A.T., Lara, E.C., Rabelo, C.H.S, Berchielli, T.T., Teixeira, I.A.M.A., Siqueira, G.R., Reis, R.A., 2014. Effects of feeding corn silage inoculated with microbial additives on the ruminal fermentation, microbial protein yield, and growth performance of lambs. *Journal of Animal Science* 92, 5640-5650.

Baurhoo, B., Mustafa, A., 2014. Short communication: Effects of molasses supplementation on performance of lactating cows fed high-alfalfa silage diets. *Journal of Dairy Science* 97, 1072-1076.

Benchaar, C., Duynisveld, J.L., Charmley, E., 2006. Effects of monensin and increasing dose levels of a mixture of essential oil compounds on intake, digestion and growth performance of beef cattle. *Journal of Animal Science* 86, 91-96.

Birollo, G.A., Reinheimer, J.A., Vinderola, C.G., 2000. Viability of lactic acid microflora in different types of yoghurt. *Food Research International* 33, 799-805.

Bonnet, M., Lagier, J.C., Raoult, D., Khelaifia, S., 2019. Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. *New Microbes and New Infections* 30, 100622.

Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., 1990. Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 28, 495-503.

Broderick, G.A., Luchini, N.D., Reynolds, S.M., Varga, G.A., Ishler, V.A., 2008. Effect on production of replacing dietary starch with sucrose in lactating dairy cows. *Journal of Dairy Science* 9, 4801-4810.

Cao, Y., Takahashi, T., Horiguchi, K.i., Yoshida, N., 2010. Effect of adding lactic acid bacteria and molasses on fermentation quality and *in vitro* ruminal digestion of total mixed ration silage prepared with whole crop rice. *Japanese Society and Grassland Science* 56, 19-25.

Cao, Y., Cai, Y., Takahashi, T., Yoshida, N., Tohno, M., Uegaki, R., Nonaka, K., Terada, F., 2011. Effect of lactic acid bacteria inoculant and beet pulp addition on fermentation characteristics and *in vitro* ruminal digestion of vegetable residue silage. *Journal of Dairy Science* 94, 3902-3912.

Charbonneau, E., Chouinard, P.Y., Allard, G., Lapierre, H., Pellerin, D., 2006. Milk from forage as affected by carbohydrate source and degradability with alfalfa silage-based diets. *Journal of Dairy Science* 89, 283-293.

Chibisa, G.E., Gorka, P., Penner, G.B., Berthiaume, R., Mutsvangwa, T., 2015. Effects of partial replacement of dietary starch from barley or corn with lactose on ruminal function, short-chain fatty acid absorption, nitrogen utilization, and production performance of dairy cows. *Journal of Dairy Science* 98, 2627-2640.

Contreras-Govea, F.E., Muck, R.E., Mertens, D.R., Weimer, P.J., 2011. Microbial inoculant effects on silage and *in vitro* ruminal fermentation, and microbial biomass estimation for alfalfa, BMR corn, and corn

silages. *Animal Feed Science and Technology* 163, 2-10.

Daniel, J.L.P., Queiroz, O.C., MArriola, K.G., Daetz, R., Basso, F., Romero, J.J., Adesogan, A.T., 2018. Effects of homolactic bacterial inoculant on the performance of lactating dairy cows. *Journal of Dairy Science* 101, 5145-5152.

Dehority, B.A., 1969. Pectin-fermenting bacteria isolated from the bovine rumen. *Journal of Bacteriology* 99, 189-196.

Devika, M., Bhuvaneshwari, K., Mohanram, K., 2019. Comparative study of membrane filtration and spread technique for dialysis water analysis. *Saudi Journal of Pathology and Microbiology* 4, 649-653.

Dougherty, D.P., Breidt, F., McFeeters, R., Lubuin, S.R., 2002. Energy-based dynamic model for variable temperature batch fermentation by *Lactococcus lactis*. *Applied and Environmental Microbiology* 68, 2468-2478.

Elam, N.A., Gleghorn, J.F., Rivera, J.D., Galyean, M.L., Defoor, P.J., Brashears, M.M., Younts-Dahl, S.M., 2003. Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacterium freudenreichii* on performance, carcass, and intestinal characteristics, and *Escherichia coli* strain O157 shedding of finishing beef steers. *Journal of Animal Science* 81, 2686-2698.

Ellis, J.L., Hindrichsen, I.K., Klop, G., Kinley, R.D., Milora, N., Bannink, A., Dijkstra, J., 2015. Effects of lactic acid bacteria silage inoculation on methane emission and productivity of Holstein Friesian dairy cattle. *Journal of Dairy Science* 99, 7159-7174.

Fernando, S.C., Purvis, H.T., Najar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja, T.G., Roe, B.A., DeSilva, U., 2010. Rumen microbial population dynamics during adaptation to a high grain diet. *Applied and Environmental Microbiology* 76, 7482-7490.

Gao, X., Oba, M., 2016. Effect of increasing dietary non-fiber carbohydrate with starch, sucrose, or lactose on rumen fermentation and productivity of lactating dairy cows. *Journal of Dairy Science* 99, 291-300.

Golder, H.M., Celi, P., Rabiee, A.R., Heuer, C., Bramley, E., Miller, D.W., King, R., Lean, I.J., 2012. Effects of grain, fructose, and histidine on ruminal pH and fermentation products during in induced subacute acidosis protocol. *Journal of Dairy Science* 95, 1971-1982.

Guo, X., Xu, D., Li, F., Bai, J., Su, R., 2022. Current approaches on the roles of lactic acid bacteria in crop silage. *Microbial Biotechnology* 16, 67-87.

Heldt, J.S., Cochran, R.C., Stokka, G.K., Farmer, C.G., Mathis, C.P., Tigemeyer, E.C., Nagaraja, T.G., 1999. Effects of different supplemental sugars and starch fed in combination with degradable intake protein on low-quality forage use by beef steers. *Journal of Animal Science* 77, 2793-2802.

Hiltner, P., Dehority, B.A., 1983. Effect of soluble carbohydrates on disappearance of cellulose by pure cultures of rumen bacteria. *Applied and Environmental Microbiology* 46, 642-648.

Khalili, H., Huhtanen, P., 1991. Sucrose supplements in cattle given silage-based diet. 2. Digestion of cell wall carbohydrates. *Animal Feed Science and Technology* 33, 263.

Kleen, J.L., Hooijer, G.A., Rehage, J., Noordhuizen, J.P., 2003. Subacute ruminal acidosis (SARA): A review. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* 50, 406-414.

Krause, K.M., Oetzel, G.R., 2005. Inducing subacute ruminal acidosis in lactating dairy cows. *Journal of Dairy Science* 88, 3633-3639.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Sjoback, R., Sjogreen, B., Strombom, L., Stahlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine* 27, 95-125.

Kung, L.J., 2006. Direct-fed microbial and enzyme feed additives. In: Direct-Fed Microbial, Enzyme and Forage Additive Compendium. Miller Publishing, Minnetonka, MN.

Kung, L., Stokes, M.R., Lin, C.J., 2003. Silage additives. In: Buxton, D.R., Muck, R.E, Harrison, J.H. (Eds.), Silage Science and Technology. American Society of Agronomy, Madison, pp. 305-360.

Lee, M.R., Merry, R.J., Davies, D.R., Moorby, J.M., Humphreys, M.O., Theodorou, M.K., MacRae, J.C., Scollan, N.D., 2003. Effect of increasing availability of water-soluble carbohydrates on *in vitro* rumen fermentation. *Animal Feed Science and Technology* 104, 59-70.

McCormick, M.E., Redfearn, D.D., Ward, J.D., Blouin, D.C., 2001. Effect of protein source and soluble carbohydrate addition on rumen fermentation and lactation performance of Holstein cows. *Journal of Dairy Science* 84, 1686 -1697.

Muck, R.E., Filya, I., Contreras- Govea, F.E., 2007. Inoculant effects on alfalfa silage: In vitro gas and volatile fatty acid production. *Journal of Dairy Science* 90, 5115-5125.

Nkosi, B.D., Meeske, R., Langa, T., Thomas, R.S., 2011. Effects of bacterial silage inoculants on whole-crop maize silage fermentation and silage digestibility in rams. *South African Journal of Animal Science* 41, 351-359.

Oba, M., 2011. Review: Effects of feeding sugars on productivity of lactating dairy cows. *Journal of Animal Science* 9, 37- 46.

Oelker, E.R., Reveneau, C., Firkins, J.L., 2009. Interaction of molasses and monensin in alfalfa hay- or corn silage- based diets on ruminal fermentation, total-

tract digestibility, and milk production by Holstein cows. *Journal of Dairy Science* 92, 270-285.

Penner, G.B., Oba, M., 2009. Increasing dietary sugar concentration may improve dry matter intake, ruminal fermentation, and productivity of dairy cows in the postpartum phase of the transition period. *Journal of Dairy Science* 92, 3341-3353.

Piwonka, E.J., Firkins, J.L., 1993. Effect of glucose on fiber digestion and particle-associated carboxymethylcellulase activity *in vitro*. *Journal of Dairy Science* 76,129-139.

Poorkasegaran, S., Yansari, A.T., 2014. Effects of different sources of carbohydrates on intake, digestibility, chewing, and performance of Holstein dairy cows. *Journal of Animal Science and Biotechnology* 5, 1-11.

Rooke, J.A., Hatfield, R.D., 2003. Biochemistry of ensiling. *Silage Science and Technology* 42, 95-139.

SAS, 2004. SAS User's Guide: Statistics. Version 9.4. SAS Institute Inc., Cary, North Carolina. USA.

Sniffen, C.J., O'Connor, J.D., Van Soest, P.J., Fox, D.G., Russell, J.B., 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *Journal of Animal Science* 70, 3562-3577.

Tang, Z., Yin, Y., Zhang, Y., Haung, R., Sun, Z., Li, T., Chu, W., Kong, X., Li, L., Geng, M., Tu, Q., 2009. Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin-lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. *British Journal of Nutrition* 101, 998-1005.

Tanhaeian, A., Mirzaii, M., Pirkhezranian, Z., Sekhavati, M.H., 2020. Generation of an engineered food-grade *Lactococcus lactis* strain for production of an antimicrobial peptide: *in vitro* and *in silico* evaluation. *BMC Biotechnology* 20, 1-13.

Vallimont, J.E., Bargo, F., Cassidy, T.W., Luchini, N.D., Broderick, G.A., Varga, G.A., 2004. Effects of replacing dietary starch with sucrose on ruminal fermentation and nitrogen metabolism in continuous culture. *Journal of Dairy Science* 87, 4221- 4229.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74, 3583-3597.

Weinberg, Z.G., Shatz, O., Chen,Y., Yosef, E., Nikhbahat, M., Ben-Ghedalia, D., Miron, I. 2007. Effect of Lactic acid bacteria inoculants on *in vitro* digestibility of wheat and corn silages. *Journal of Dairy Science*, 90, 4754-4762.

Weisbjerg, M.R., Hvelplund, T., Bibby, B.M., 1998. Hydrolysis and fermentation rate of glucose, sucrose and lactose in the rumen. *Acta Agriculturae Scandinavica- Section A: Animal Science* 48, 12-18.

Yoon, I.K., Stern, M.D., 1995. Influence of DFM on ruminal microbial fermentation and performance of ruminants: A Review. *Asian – Australasian Journal of Animal Sciences* 8, 533-555.