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Original article

Milk production and ruminal parameters of dairy cows fed diets containing *Lactobacillus sakei* KTU05-6 and *Pediococcus pentosaceus* BaltBio02

V. Lele, R. Zelvyte, I. Monkeviciene, J. Kantautaite, R. Stankevicius, M. Ruzauskas, A. Sederevicius, R. Antanaitis, E. Bartkiene

Lithuanian University of Health Sciences, Tilzes str. 18, 47181 Kaunas, Lithuania

Abstract

Effects of fermented extruded rye flour supplements with *Lactobacillus sakei* KTU05-6 or *Pediococcus pentosaceus* BaltBio02 on milk production and composition, as well as ruminal parameters, were determined in Lithuanian Black & White dairy cows. Also, determination of antimicrobial activities of tested lactic acid bacteria (LAB) against a variety of pathogenic and opportunistic bacterial strains previously isolated from diseased cattle was performed. The highest antimicrobial activity was demonstrated in *L. sakei* against *S. aureus*, and in *P. pentosaceus* against *P. aeruginosa* and *S. aureus*. The count of LAB in the supplements after 72 h of fermentation of extruded rye flour with *L. sakei* and *P. pentosaceus* was 9.6±0.4 $\log_{10}$ CFU/g and 9.5±0.3 $\log_{10}$ CFU/g, respectively. All cows (n=60) were fed the same basal diet. The treatment differences were achieved by individually incorporating (65 d.) one of the supplements: *L. sakei* KTU05-6 (group B; n=20) or *P. pentosaceus* BaltBio02 (group C; n=20). The control group A (n=20) was on the basal diet only. A supplement fermented with *L. sakei* does not have a significant influence on dairy cattle milk production and rumen fluid parameters. The type of LAB used has a significant influence ($p<0.0001$) on microbiological parameters of the rumen (TCM, TCL, TCE). The milk yield was increased ($p\leq0.05$) using *P. pentosaceus* BaltBio02 supplement, and further research is needed to identify what is the main mechanism of the positive action.

Key words: dairy cows, lactic acid bacteria, antimicrobial activities, rumen, milk

Introduction

An alternative and effective approach to antibiotic administration to livestock is the use of lactic acid bacteria (LAB), which can help to improve gut microbial balance and therefore the natural defence of the animal against pathogenic bacteria (Patterson and Burkholder 2003). Commonly used bacteria include various species of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* as well as some *Enterococcus* species (Morrow et al. 2012). Because of the LAB antimicrobial effect and their production of bacteriocin-like inhibitory substances

Correspondence to: R. Antanaitis, e-mail: ramunas.antanaitis@lsmuni.lt, tel.: +370 67349064

(BLIS), they have the potential to be used as modifiers of the ecosystem in the gastrointestinal tract (Seo et al. 2010) and stimulators of animal production (Yasuda et al. 2007). However, the effect of LAB is variable, even within the same species (Gobbetti et al. 1999). Ruminants rely on a symbiosis between the host and the rumen microbes, the microorganisms supply protein, vitamins and short chain organic acids for the animal host. The energy absorbed, glucose formation in the liver, and the protein digested in the gastric stomach (the abomasum) are all mainly derived from microbial origins (Pinloche et al. 2013). Given the importance of the microbial population in feed conversion, it is perhaps not surprising that a great many studies have investigated the possibility of manipulating rumen fermentation to boost animal productivity (Puniya et al. 2015, Yanez-Ruiz et al. 2015).

It is widely expected that in cattle the performance/health state of individuals will be linked to characteristic transitions in the rumen microbiota, and links have already been made between certain nutritional derived pathologies (acidosis, laminitis) and alteration of the microbiota of the rumen (Lima et al. 2009). *L. sakei* and *P. pentosaceus* produced BLIS show wide-ranging antimicrobial activities against gram positive and gram negative strains (Cizeikiene et al. 2013). However, studies on the possible effect of *L. sakei* and *P. pentosaceus* feeding on ruminants are scarce.

The aim of this study was to investigate the influence of *Lactobacillus sakei* KTU05-6 and *Pediococcus pentosaceus* BaltBio02 on dairy cow milk production and milk composition as well as ruminal parameters. Determination of antimicrobial activities of tested LAB against a variety of pathogenic and opportunistic bacterial strains previously isolated from diseased cattle was also performed.

Materials and Methods

Evaluation of antimicrobial activities of *L. sakei KTU05-6* and *P. pentosaceus BaltBio02*

The LAB were grown in de Man Rogosa Sharpe (MRS) medium (Biolife, Italy) at their optimal temperatures of 30°C (*Lactobacillus sakei*), or 35°C (*Pediococcus pentosaceus*). Two percent of LAB cells (v/v) were inoculated into a fresh medium and propagated for 18 h. The cells were harvested by centrifugation (6000 g, 10 min, 4°C). The culture supernatants were filtered through a 0.2 mm sterile Millipore filter to remove all cells. Supernatants were used for the determination of antimicrobial activities of *L. sakei* and *P. pentosaceus* strains against a variety of pathogenic and opportunistic bacterial strains (*Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Salmonella enterica; Corynebacter spp; Klebsiella pneomoniae, Enterococcus faecalis,* and *Bacillus cereus*) previously isolated from diseased cattle. Agar well diffusion assay was used for antimicrobial activities testing. For this purpose, a 0.5 McFarland Unit density suspension of each pathogenic bacterial strain was inoculated onto the surface of cooled Mueller Hinton Agar (Oxoid, UK) using sterile cotton swabs. Wells of 6 mm in diameter were punched in the agar and filled with 50 µl of LAB supernatants. The experiments were repeated three times, and the average of inhibition zones was calculated. The antimicrobial activities against tested bacteria were determined by measuring the diameter of inhibition zones (mm).

Preparation of extruded rye flour supplements with high amount of *L. sakei KTU05-6* and *P. pentosaceus BaltBio02*

Extrusion of rye wholemeal was used for flour thermal treatment with the intention of reducing the level of microbial contamination. Extruded rye wholemeal (moisture content 8.6%) producing a single-screw extruder (Ustukiu Malunas Ltd, Pasvalys, Lithuania) was used as a cultivation medium for *Lactobacillus sakei* KTU05-6 and *P. pentosaceus* BaltBio02. The LAB used in the experiment were isolated from spontaneous fermented rye by additionally proving their bacteriocin activity (Digaitiene et al. 2012). The strains were stored at -80°C in a Microbank system (PRO-LAB DIAGNOSTICS) and were propagated in MRS broth (CM 0359, Oxoid Ltd, Hampshire, UK) at 30°C for 48 hours with the addition of 40 mM fructose and 20 mM maltose prior to use. The bacteria were diluted with a physiological saline (0.9 % sterile solution of sodium chloride in water) to a concentration of 9 log10 CFU/mL before the rye wholemeal fermentation.

3.0 kg of extruded rye wholemeal flour, 4.5 L of water, and 50 mL LAB cell suspension (containing about 9 log10 CFU/mL) were mixed and fermented for 72 hours at a temperature optimal for the strains (*L. sakei* – 30°C; *P. pentosaceus* – 35°C). The supplements were divided into 100 g pieces and used for cows feeding. As the LAB count during the 7-day period was not lower than 9 $\log_{10}$ CFU/g (storage temperature +4°C), a new portion of fermented supplement was prepared every week.

Microbiological analysis of the fermented supplements

For microbiological analysis, 10 g of the sample was homogenized with 90 mL of saline (0.9%). The suspen-

Table 1. Ingredients and nutrient composition of the diets fed to dairy cows.

Diet composition	Weight, kg		
	Group A[a]	Group B[a]	Group C[a]
Haylage	22.0	22.0	22.0
Triticale	7.0	7.0	7.0
Urea	0.055	0.055	0.055
Mineral supplement	0.200	0.200	0.200
L. sakei supplement	-	0.100	-
P. pentosaceus supplement	-	-	0.100
	Nutrient content		
Dry matter, kg	17.3	17.4	17.42
NEL, MJ	114.3	114.9	114.8
Crude protein, g	2514.0	2522.0	2529.0
Crude fibre, kg	3.3	3.4	3.00
Usable crude protein, g	2438.0	2450.0	2458.0
Ruminal ammonia balance, g	12.1	11.7	11.9
Ca, g	108.7	108.8	108.6
P, g	67.1	67.4	67.9
Ca:P	1.62:1	1.61:1	1.61:1

[a] group A = control (basal diet); group B = basal diet plus supplement with *L. sakei*; group C = basal diet plus supplement with *P. pentosaceus*.
NEL = net energy for lactation; Ca = Calcium; P = Phosphorus

sion was diluted, and the 10^{-4}–10^{-8} solutions were inoculated in MRS agar (CM0361, Oxoid Ltd, Hampshire, UK) and incubated under anaerobic conditions at 35°C for 72 hours (for LAB). MacConkey agar (CM0007B; Oxoid Ltd, Hampshire, UK) was used for determination of the total count of enterobacteria; the Nutrient agar (Biolife Italiana Srl, Milan, Italy) was used for the total aerobic bacteria, and the Sabouraud glucose agar (C974Q82, Sigma-Aldrich, Germany) was used for fungi, following incubation under aerobic conditions at 37°C for 72 hours. The final number of bacteria was calculated and expressed as a log10 of colony forming units (CFU) per gram of the sample. Three replications per treatment were prepared.

Animals, diets and experimental procedures

Sixty clinically healthy dairy cows of Lithuanian Black and White breed were used in this study. The cows were in mid-lactation and had body weights of 501±2.5 kg.

All cows were fed the same basal diet (Table 1) twice a day at 07.00 and 18.00 h and had unlimited access to fresh water. The treatment differences were achieved by individually incorporating one of the supplements: *L. sakei* KTU05-6 (group B; n=20) or *P. pentosaceus* BaltBio02 (group C; n=20). The supplements were added once daily immediately after the morning feed delivery for 65 days. The control group A (n=20) was on the basal diet only.

The cows were hosted indoors, individually tethered, and cared for in accordance with the "Requirements for keeping, maintenance and use of animals intended for experimental and other scientific purposes".

Milk sample collection and analyses

All cows (n=60) were milked twice a day at 05.00 and 16.00 h. Samples of the milk were taken: (d 0) one day before the experiment; at the 33rd day of treatment (d 33) and the next day after the last treatment (d 66).

Milk yields for all cows were determined using *Tru-test milk meters* (Tru-test Ltd; New Zealand) during sampling days. The milk samples were collected in 50 mL vials, preserved with *Sedupol* (8 mg bronopol and 0.3 mg natamicin/tab), stored at +4°C, and analysed for milk components (fat, protein, lactose, urea) by near infrared analyses using a *LactoScope FTIR* (FT1.0. 2001; Delta Instruments, Holland).

Sampling and analyses of rumen fluid

Approximately 500 ml of rumen fluid was collected from 15 randomly selected animals (five from each group) at d 0 and d 66. The collections of rumen fluid were conducted using a stomach tube 3 hours after the morning feed. Ruminal pH was measured immediately after the sampling, using a handheld pH-meter (Horiba - Twin pH, Spectrum Technologies, Japan). The rumen fluid was strained through four layers of cheesecloth for

Table 2. Inhibition of the growth of pathogenic bacteria by *Lactobacillus sakei* and *Pediococcus pentosaceus.*

Microorganisms	Zone of inhibition/mm			
	L. sakei		*P. pentosaceus*	
	Mean	STDEV	Mean	STDEV
P. aeruginosa	15.3	0.4	17.3	0.6
S. aureus	19.8	0.6	16.4	0.5
E. coli	14.2	0.3	13.7	0.3
S. enterica	11.5	0.4	11.3	0.3
Corynebacterium spp.	12.3	0.2	12.7	0.4
K. pneumoniae	10.6	0.3	11.6	0.6
E. faecalis	13.7	0.4	13.3	0.3
B. cereus	12.1	0.5	14.5	0.2

Values are mean of three replicate analyses.
STDEV = standard deviation of the mean

Table 3. Microbiological parameters of supplements fermented with *L. sakei* and *P. pentosaceus.*

Samples	Fermentation time, h	LAB		Total aerobic bacteria		Enterobacteria		Fungi			
								yeast		mold	
		$\log_{10}$ CFU/g									
		Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Untreated	0	4.6a	0.1	5.4c	0.1	5.9	0.2	6.1d	0.2	4.6b	0.1
L. sakei	24	7.6b	0.2	4.5b	0.3	-	-	5.6c	0.2	4.3a	0.3
	48	8.9d	0.2	4.6b	0.2	-	-	5.2b	0.1	4.2a	0.1
	72	9.5d	0.3	4.6b	0.1	-	-	5.1a	0.3	4.2a	0.1
P. pentosaceus	24	8.4b	0.3	4.4b	0.2	-	-	5.7c	0.3	4.4a	0.2
	48	9.3d	0.2	4.6b	0.4	-	-	5.4b	0.4	4.3a	0.3
	72	9.6d	0.4	4.6b	0.3	-	-	5.2a	0.2	4.3a	0.2

Values are mean of three replicate analyses.
Different letters indicate significant differences between mean values of treatments ($p<0.05$)
L. sakei = fermented with *L. sakei*; *P. pentosaceus* = fermented with *P. pentosaceus*; LAB = lactic acid bacteria, SSF = solid state fermentation, CFU = colony forming units; STDEV = standard deviation of the mean

volatile fatty acids (VFA), total nitrogen (N), ammonia nitrogen (NH_3-N), L(+)- and D(-)-lactate analysis. Non-strained samples of rumen fluid were used for protozoa number, total count of aerobic and facultative anaerobic microorganisms (TCM), total count of lactobacilli (TCL) and enterobacteria (TCE) analysis.

The analyses of VFA were conducted by gas chromatography (GC-2010 Shimadzu, Japan). Total N was analysed using the Kjeldahl procedure (Behr system, Germany), NH_3-N – using a titrimetric method with preliminary distillation (Behr steam distillation unit S1, Germany). The concentrations of L(+)- and D(-)-lactic acid were evaluated using an enzyme test kit (R-biopharm AG - Roche, Darmstadt, Germany), as reported elsewhere (Lima et al., 2009).

A Fuchs-Rosenthal counting chamber (Blaubrand, Wertheim, Germany) was used for enumeration of protozoa using an *Olympus* microscope (BX43, Hamburg, Germany). The total count of aerobic and facultative anaerobic microorganisms was determined on Plate count agar (CM0325, Oxoid, UK) after incubation at 30°C for 72 hours. Analysis of viable LAB in the rumen fluid samples was performed under anaerobic conditions using an atmosphere generation system *AnaeroGen* (Oxoid, Basingstoke, UK). For detection of the total number (log10 CFU/mL) of enterobacteria, the diluted samples were placed on MacConkey agar (CM0007, Oxoid, UK).

Statistical analyses

The experimental data were evaluated statistically and presented as mean, standard deviation of the mean (STDEV) for inhibition of the growth of pathogenic bacteria by *L. sakei* and *P. pentosaceus* and microbiological parameters of the supplements, and as mean, standard error of the mean (SEM) for milk and rumen fluid parameters. In order to evaluate the influence of different factors (type of LAB and supplemental feeding period) and their interaction on dairy cattle milk

Table 4. Influence of *L. sakei* and *P. pentosaceus* supplements on feed intake, milk production and milk composition of dairy cows.

	Group A[a]		Group B[a]		Group C[a]		*p*-value[b]				
	Mean	SEM	Mean	SEM	Mean	SEM	A *vs.* B	A *vs.* C	B *vs.* C	B d0 *vs.* d33 / B d0 *vs.* d66	C d0 *vs.* d33 / C d0 *vs.* d66
Daily feed intake											
Total (kg DM)	16.81	0.09	16.94	0.08	16.98	0.08	0.26	0.15	0.74	-	-
Milk parameters											
DAY 0											
Yield, kg/d	18.74	0.94	17.78	1.51	18.08	0.89	0.60	0.62	0.87	-	-
4% FCM, kg/d	20.23	1.12	19.16	1.38	19.19	1.14	0.55	0.52	0.99	-	-
Fat, %	4.53	0.18	4.64	0.33	4.42	0.13	0.77	0.64	0.56	-	-
Protein, %	3.11	0.15	3.39	0.18	3.29	0.14	0.26	0.40	0.67	-	-
Lactose, %	4.46	0.09	4.59	0.06	4.45	0.05	0.26	0.95	0.09	-	-
Urea, mg/dL	17.1	1.64	18.1	1.8	15.33	1.39	0.69	0.43	0.25	-	-
DAY 33											
Yield, kg/d	18.2	0.94	17.46	1.31	18.87	1.57	0.65	0.71	0.50	0.87	0.67
4% FCM, kg/d	19.63	0.70	19.97	1.54	19.88	1.56	0.84	0.88	0.97	0.70	0.72
Fat, %	4.58	0.17	5.03	0.41	4.42	0.26	0.33	0.61	0.24	0.47	0.99
Protein, %	3.43	0.18	3.69	0.19	3.23	0.19	0.33	0.45	0.10	0.27	0.17
Lactose, %	4.49	0.07	4.61	0.07	4.54	0.04	0.25	0.58	0.41	0.81	0.17
Urea, mg/dL	10.2	0.70	11.4	1.51	11.44	1.13	0.48	0.35	0.98	0.06	0.06
DAY 66											
Yield, kg/d	16.44	0.99	15.72	1.33	22.49	1.58	0.67	0.004	0.004	0.32	0.03
4% FCM, kg/d	17.81	0.85	17.7	1.16	23.53	1.34	0.94	0.002	0.004	0.43	0.02
Fat, %	4.63	0.21	4.97	0.32	4.45	0.26	0.39	0.58	0.23	0.49	0.94
Protein, %	3.45	0.14	3.62	0.19	3.48	0.14	0.48	0.88	0.58	0.40	0.35
Lactose, %	4.46	0.06	4.60	0.04	4.45	0.04	0.07	0.81	0.02	0.86	0.95
Urea, mg/dL	17.2	1.56	16.4	1.36	15.78	0.74	0.70	0.44	0.70	0.46	0.78

[a] group A = control (basal diet); group B = basal diet plus supplement with *L. sakei*; group C = basal diet plus supplement with *P. pentosaceus*.
[b] *p*-value contrast: A *vs.* B = group A *vs.* group B; A *vs.* C = group A *vs.* group C; B *vs.* C = group B *vs.* group C; B d0 *vs.* d33 / B d0 *vs.* d66 = day 0 *vs.* day 33 and day 0 *vs.* day 66 in group B; C d 0 *vs.* d 33 / C d0 *vs.* d 66 = day 0 *vs.* day 33 and day 0 *vs.* day 66 in group C. FCM = 4% fat-corrected milk; SEM = standard error of the mean.

production and rumen fluid parameters, the data were subjected to Univariate Analysis of Variance (ANOVA, statistical program R 3.2.1, R Core Team 2015) and Post Hoc Tests with a 95% confidence Interval (for Homogeneous Subsets Tukey HSD was used) was performed. The results were considered to be statistically significant at $p \leq 0.05$.

Results

Antimicrobial activities of *L. sakei* and *P. pentosaceus* and microbiological parameters of supplements

The antimicrobial activities of the LAB are presented in Table 2. As can be seen from the obtained results, LAB supernatants inhibited the growth of all tested bacteria. The highest antimicrobial activity was demonstrated by *L. sakei* against *S. aureus* (inhibition zone diameter was 19.8 ± 0.6 mm). The lowest inhibition zones were observed against *K. pneumoniae* and *S. enterica* (10.6 ± 0.3 mm and 11.5 ± 0.4 mm, respectively). The highest diameters of the inhibition zones of *P. pentosaceus* toward pathogenic strains were 17.3 ± 0.6 mm and 16.4 ± 0.5 mm (against *P. aeruginosa* and *S. aureus*, respectively).

The microbiological results of the fermented supplements are presented in Table 3. The count of LAB in the samples after 72 h of fermentation with *L. sakei* was 9.5±0.3 log10 CFU/g, and after 72 h of fermentation with *P. pentosaceus* was 9.6±0.4 log10 CFU/g. Fermentation inhibited the growth of *Enterobacteriaceae* and reduced the growth of aerobic bacteria and yeast in rye substrate. Reduction of mould growth in the fermented

Table 5. Effect of *L. sakei* and *P. pentosaceus* supplements on biochemical and microbiological parameters of rumen fluid of dairy cows.

Parameters	Group A[a]		Group B[a]		Group C[a]		*p*-value[b]				
	Mean	SEM	Mean	SEM	Mean	SEM	A *vs.* B	A *vs.* C	B *vs.* C	B d0 *vs.* d66	C d0 *vs.* d66
DAY 0											
pH	6.62	0.06	6.40	0.23	6.48	0.02	0.46	0.06	0.69	-	-
Total VFA, mmol/L	117.0	5.00	120.0	1.05	103.7	6.75	0.62	0.26	0.16	-	-
Relative proportion of VFA, %:											
Acetate	71.61	0.54	68.37	1.48	69.33	1.54	0.11	0.24	0.68	-	-
Propionate	13.84	0.62	14.96	0.55	14.2	0.44	0.25	0.66	0.34	-	-
Butyrate	12.55	1.25	14.59	0.77	14.06	0.48	0.24	0.43	0.65	-	-
Isobutyrate	0.30	0.08	0.32	0.05	0.36	0.05	0.86	0.59	0.59	-	-
Valerate	0.97	0.13	1.11	0.06	1.25	0.25	0.48	0.35	0.65	-	-
Isovalerate	0.30	0.05	0.23	0.12	0.38	0.11	0.55	0.53	0.46	-	-
Caproate	0.45	0.13	0.43	0.06	0.44	0.04	0.90	0.94	0.90	-	-
A/P	5.18	0.16	4.58	0.15	4.91	0.39	0.06	0.50	0.42	-	-
Total N, mg/dL	75.25	7.35	61.95	8.75	62.65	10.15	0.36	0.42	0.96	-	-
NH_3-N, mg/dL	7.17	1.35	7.15	0.77	9.8	0.62	0.99	0.22	0.11	-	-
L(+)-lactic acid, mmol/L	0.49	0.15	0.38	0.05	0.21	0.02	0.54	0.20	0.11	-	-
D(-)-lactic acid, mmol/L	<0.001		<0.001		<0.001						
Protozoa number, $\times 10^5$/mL	2.27	0.30	2.12	0.26	1.71	0.34	0.74	0.27	0.37	-	-
TCM, $\log_{10}$ CFU/mL	5.10	0.21	5.09	0.27	6.17	0.24	0.99	0.06	0.06	-	-
TCL, $\log_{10}$ CFU/mL	5.24	0.07	5.31	0.14	5.83	0.18	0.68	0.08	0.13	-	-
TCE, $\log_{10}$ CFU/mL	2.22	0.15	1.47	0.11	2.33	0.20	0.06	0.70	0.06	-	-
DAY 66											
pH	6.21	0.29	6.35	0.18	6.62	0.09	0.70	0.25	0.26	0.86	0.19
Total VFA, mmol/L	143.3	6.67	137.5	4.79	119.0	6.08	0.50	0.06	0.06	0.07	0.17
Relative proportion of VFA, %:											
Acetate	70.92	1.46	68.92	1.09	72.83	2.23	0.31	0.51	0.12	0.77	0.27
Propionate	14.51	0.83	14.52	0.73	15.33	0.23	0.99	0.40	0.45	0.69	0.09
Butyrate	12.25	1.28	12.85	0.76	12.54	1.19	0.68	0.88	0.82	0.18	0.40
Isobutyrate	0.40	0.06	0.37	0.05	0.43	0.12	0.66	0.83	0.56	0.54	0.67
Valerate	0.98	0.05	1.04	0.06	1.83	0.22	0.47	0.41	0.46	0.55	0.87
Isovalerate	0.45	0.04	0.47	0.09	0.49	0.10	0.85	0.71	0.87	0.16	0.50
Caproate	0.50	0.05	0.47	0.04	0.54	0.15	0.69	0.82	0.60	0.61	0.64
A/P	4.89	0.17	4.86	0.14	4.75	0.17	0.92	0.60	0.63	0.24	0.70
Total N, mg/dL	77.47	11.32	70.52	9.39	78.17	9.22	0.65	0.96	0.60	0.59	0.35
NH_3-N, mg/dL	6.50	0.36	7.28	1.19	9.11	0.71	0.56	0.06	0.26	0.94	0.55
L(+)-lactic acid, mmol/L	0.18	0.09	0.10	0.04	0.17	0.11	0.40	0.96	0.53	0.02	0.81
D(-)-lactic acid, mmol/L	<0.001		<0.001		<0.001						
Protozoa number, $\times 10^5$/mL	2.34	0.48	2.24	0.47	2.51	0.16	0.89	0.75	0.65	0.84	0.10
TCM, $\log_{10}$ CFU/mL	5.70	0.22	6.34	0.14	6.97	0.23	0.04	0.02	0.06	0.009	0.08
TCL, $\log_{10}$ CFU/mL	4.70	0.07	4.92	0.19	6.93	0.20	0.39	0.0005	0.0008	0.25	0.01
TCE, $\log_{10}$ CFU/mL	2.20	0.09	1.87	0.19	2.13	0.15	0.22	0.72	0.34	0.24	0.47

[a] group A = control (basal diet); group B = basal diet plus supplement with *L. sakei*; group C = basal diet plus supplement with *P. pentosaceus*.

[b] *p*-value contrast: A *vs.* B = group A *vs.* group B; A *vs.* C = group A *vs.* group C; B *vs.* C = group B *vs.* group C; B d0 *vs.* d66 = day 0 *vs.* day 66 in group B; C d0 *vs.* d66 = day 0 *vs.* day 66 in group C.

VFA = volatile fatty acids; A/P = acetate to propionate ratio; N = nitrogen NH_3-N = ammonia nitrogen; TCM = total count of aerobic and facultative anaerobic microorganisms; TCL = total count of lactobacilli; TCE = total count of enterobacteria; SEM = standard error of the mean

cereal samples was lower compared to that of the yeast, but statistically significant ($p \leq 0.05$).

Effect of *L. sakei* and *P. pentosaceus* supplements on milk production and composition

At the beginning (d 0) of the experiment, no statistically significant differences were observed in any of the evaluated production parameters between the control and treated groups (Table 4). The milk yield declined steadily (d33; d66) in all groups during the study. After 65 days of feeding with *P. pentosaceus* supplement (group C), the milk yield increased and was statistically significantly higher in comparison with the control group and Group B ($p<0.05$). At the end of the experiment (d66), the fat-corrected milk yield decreased in groups A and B as it increased statistically significantly in group C. The results of the ANOVA test indicated that there was a significant effect on the type of LAB used for the cattle feed on milk yield ($p=0.001$), FCM ($p<0.012$), fat ($p<0.007$), protein ($p<0.002$), and lactose ($p<0.0001$) content in milk. A significant effect on the supplemental feeding period was also found on protein ($p<0.004$) and urea ($p<0.0001$) content in milk. The interaction between the analysed factors (type of LAB and supplemental feeding period) was determined as statistically significant on the milk yield ($p<0.001$) and FCM ($p<0.0001$).

The effect of the *L. sakei* and *P. pentosaceus* supplements on the rumen fluid parameters

It was found that feeding dairy cows (Group C) with *P. pentosaceus* supplement increased TCM ($p<0.05$) and TCL ($p<0.001$) in rumen fluid in comparison with Control Group A at end of the experiment (d 66) (Table 5). Although an increase in TCL ($p=0.01$) after the feeding period (d 66) was detected, the concentration of the lactic acid was not affected in Group C.

The *L. sakei* supplement had a significant influence on the L(+)-lactate concentration in rumen fluid (after 65 days of feeding the concentration decreased by 0.28 mmol/L, $p<0.05$). At the end of the experiment (d 66), TCM increased by 1.25 log10 CFU/mL ($p<0.05$) in comparison with d 0 in Group B and 0.64 log10 CFU/mL ($p>0.05$) in comparison with Control Group A.

No other significant changes in ruminal fermentation and microbiological parameters were detected; fluctuations were in normal ranges during the experimental (d 0–d 66) period ($p>0.05$).

A significant effect of the duration of feeding with supplements and type of LAB used for the dairy cow feed was found on ruminal VFA content ($p=0.001$). The type of LAB had a significant effect on acetate and valerate content in the rumen ($p<0.034$ and $p<0.0001$, respectively). A significant effect of the used LAB was also found on NH3-N and L-lactate in rumen fluid ($p<0.001$ and $p<0.043$, respectively).

The type of LAB used had a significant influence ($p<0.0001$) on the microbiological parameters of the rumen (TCM, TCL, TCE). The interaction between the analysed factors (type of LAB and supplemental feeding period) was determined as statistically significant for certain ruminal parameters (propionate ($p<0.020$), valerate ($p<0.007$), L-lactate ($p<0.042$) content, TCM ($p<0.0001$), TCL ($p<0.0001$), and TCE ($p<0.007$)).

Discussion

One of the most important criteria formulated by the World Health Organization/Food and Agriculture Organization of the United Nations (WHO/FAO) for selection of microorganisms for a probiotic purpose is their ability to display antimicrobial activity against pathogenic bacteria. Lactic acid bacteria can produce antimicrobial agents that exert strong antagonistic activity against many microorganisms, including pathogenic and spoilage microorganisms (Adeniyi et al. 2015). Metabolites, such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetoine, carbon dioxide, reuterin, reutericyclin and bacteriocins, are examples of antimicrobial agents produced by LAB (Jagoda et al. 2010). The antimicrobial effect of acids is due to the fact that undissociated acids can pass through the microbial lipid membranes and disrupt the proton-motive force of the host cell (Oliveira et al. 2015). Spontaneously fermented cereals represent a source of LAB with potential interesting functional properties as well as a potential source of probiotics. In this study, the LAB used were isolated from spontaneous fermented cereals, and therefore they showed a good growth potential in the extruded rye substrate. The best nutritional characteristics of fermented cereals are the number of LAB above 9 $\log_{10}$ CFU/g, pH lower than 4.5, and lactic acid concentration higher than 13.5 g/L (Missotten et al. 2013). This study showed that the recommended count of LAB cells could be achieved after 72 h of fermentation. Different species belonging to the LAB group of microorganisms are increasingly gaining attention as a possible means of inhibiting mould growth in animal feed chains (Hassan et al. 2016). Lactic acid bacteria antifungal capacity is a function of the substrate and growth conditions applied, among which, the fermentation time has an essential role on the production of bacterial metabolites (Zuo et al. 2012). Lactic acid bacteria efficacy relies on the production of a wide range of antifungal and antimicrobial compounds (Tchikoua et al. 2015).

Different antifungal compounds have been detected within the different LAB species, which suggests that the production of LAB metabolites is species and possibly strain dependent (Oliveira et al. 2015). In our previous studies, BLIS produced by *L. sakei* KTU05-6 and *P. pentosaceus* BaltBio02 were designated as sakacin 05-6 and pediocin 05-9 (Cizeikiene et al. 2013). However, despite the promising antimicrobial properties of such microorganisms, the *in vivo* effect should be evaluated.

Milk yield, milk fat and protein production in dairy cows are economically the most valuable properties. Many studies have demonstrated the beneficial effects of LAB supplements on ruminant performance (Nagpal et al. 2015, Tristant and Moran 2015). It was found that supplementation of 100 g/cow/day of *L. sakei* (containing on average 9.6 $\log_{10}$ CFU/g) does not have an influence on milk yield and milk composition; therefore, a positive effect could be achieved by using the same amount and concentration of *P. pentosaceus* supplement. According to Weinberg et al. (2004), this might result from their probiotic effects. In clinical trials, probiotics have been reported to enhance the growth of many domestic animals including cows, neonatal calves and piglets (Shreedhar et al. 2016). Therefore, improving the productivity of the animals is important. The use of rumen manipulators is an option to enhance animal productivity. Rumen manipulation can be done by the use of many growth stimulants including hormones and antibiotics. However, this has potential risks for public health. In recent years, many LAB were widely used as rumen microecological regulators to increase production in ruminant animals. The probiotic potential of different bacterial strains, even within the same species, differs (Soccol et al. 2010). Certain LAB strains interact with rumen microorganisms to enhance rumen functionality and animal performance (Uyeno et al. 2015). The action of LAB in the rumen would yield benefits such as modified fermentation patterns, which is directly linked to improved animal performance (McAllister et al. 2011). According to Welkie et al. (2010), ruminal bacteria are vital to the health and productivity of the host. In the present study, the ruminal pH, total VFA, total N, NH_3-N, D(-)-lactic acid, protozoa number, TCL and TCE did not show any differences between Group B and the Control Group. Yet the ruminal environment was not affected by the interaction of period and supplementation with 100 g/cow/day *L. sakei* or *P. pentosaceus* (containing on average 9.6 $\log_{10}$ CFU/g) in this study.

The concentration of the L(+)-lactate decreased to 26.3% ($p<0.05$) after consumption of the supplement with *L. sakei*. However, this parameter also decreased to 36.7% ($p>0.05$) in the Control Group. Previously presented data does not support the observations of lactic acid production reported by Fayol-Messaoudi et al. (2005). They showed that *Lactobacillus* strains all produce L(+)-lactic acid while *L. sakei* forms D, L-lactic acid. This could be related to changes in the ruminal ecosystem. This ecosystem fluctuates in terms of microbial composition and environmental parameters according to dietary supplies (Monteils et al. 2011). In the present study, the *L. sakei* supplement had a statistically significant effect on TCM, and at the end of the experiment (d 66) it had increased by 24.6%. The microbial community plays a key role in digestion, producing VFA, ammonia that are directly utilised by the ruminant (Fonty and Chaucheyras-Duran 2008). Bacteria represent the majority of the microbial community in terms of biomass and fermentative activity, and bacteria numbers depend on dietary change or on the addition of feed supplements (Monteils et al. 2012).

The LAB used in this experiment showed similar antimicrobial activities but the influence on rumen parameters and milk production was different. Therefore, further research is needed to evaluate the probiotic properties of the *P. pentosaceus* BaltBio02 used in the experiment to determine if this bacteriocinogenic strain can be used as a supplement for ruminants to improve milk production and rumen fluid parameters. Supplementation of the dairy cow ration with *P. pentosaceus* BaltBio02 (9.6 $\log_{10}$ CFU/g /cow/day) increased ($p<0.05$) milk yield, yet it did not affect the milk composition (fat, protein, lactose and urea) or the rumen fermentation parameters. No impact on milk or ruminal parameters of *Lactobacillus sakei* KTU 05-6 (9.6 $\log_{10}$ CFU/g /cow/day) supplement was detected in the present study.

Conclusions

Both LAB used in the experiment showed similar antimicrobial activities against pathogenic strains; however, the milk yield was increased ($p<0.05$) by feeding supplement with a high content of *P. pentosaceus* BaltBio02, and no positive influence of *L. sakei* on the milk production and rumen fermentation parameters was observed. Hence, further research is needed to identify the main mechanism of the positive action of the *P. pentosaceus BaltBio02* supplement.

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