Effects of probiotics on in vitro ruminal profile and population of some cellulolytic bacteria

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Abstract: The aim of this study was to evaluate the effects of probiotics on ruminal pH, ammonia nitrogen, production of short chain fatty acids (SCFA) and number of *Ruminococcus albus* and *Ruminococcus flavefaciens*. The probiotics blend was tested *in vitro* for 48 h incubation using "Hohenheim Futterwert Test" (HFT). There was no interaction effect of the treatment and incubation time on the ruminal pH and ammonia-nitrogen concentration. A supplemental probiotics blend tended to increase the total SCFA concentration in comparison to the non-supplemented control fermenter fluids (P> 0.05). There was a significant interaction effect of the treatment groups and incubation time on butyrate (P=0.042), valerate (P=0.045) and *iso*valerate (P= 0.028) concentrations. Total protozoa and total bacteria numbers were higher in probiotics supplemented fluid than in non-supplemented control fluid (P<0.001 and P<0.01, respectively). Dietary supplementation of a probiotics blend to the fermenters did not influence (P>0.05) the number of copies of *R. albus*. However, the population of *R. flavafaciens* was lower (P<0.01) in the probiotic supplemented group as compared with the non-supplemented control group. Our results showed that the probiotics blend might modulate both microbial metabolic activity and the population of ruminal microorganisms.

Keywords: Cellulolytic bacteria, nutrigenomics, probiotics, ruminomics, short chain fatty acids

Probiyotiklerin in vitro rumen profili ve bazı selülolitik bakterilerin popülasyonu üzerine etkileri

Özet: Bu çalışmanın amacı probiyotiklerin ruminal pH, amonyak azotu, kısa zincirli yağ asitlerinin üretimi (SCFA) ve *Ruminococcus albus* ve *Ruminococcus flavefaciens*'in popülasyonu üzerindeki etkisini belirlemektir. Probiyotik karışımı 48 saat inkubasyonda "Hohenheim Futterwert Testi" (HFT) kullanılarak test edilmiştir. Ruminal pH ve amonyak-azot konsantrasyonu bakımından deneme grupları ve inkubasyon süresi arasında anlamlı bir etkileşim gözlenmemiştir. Probiyotik karışımı ilave edilen grup, kontrol grubuna kıyasla toplam SCFA konsantrasyonunu arttırma eğilimindeyken, gruplar arasındaki etkileşim istatistiki bakımdan önemli bulunmamıştır (P>0,05). Deneme grupları ve inkubasyon süreleri arasında bütirat (P=0,042), valerat (P=0,045) ve izovalerat (P=0,028) konsantrasyonlarında anlamlı bir etkileşim gözlenmiştir. Probiyotik ilave edilen grupta toplam protozoa ve toplam bakteri sayısının, kontrol grubundan daha yüksek olduğu gözlemlenmiştir (sırasıyla P<0,001 ve P<0,01). Fermenterlere bir probiyotik takviyesi, *R. albus*'un gen kopya sayısını etkilememiştir (P>0,05). Bununla birlikte, probiyotik takviyeli grupta, takviye edilmemiş kontrol grubuyla karşılaştırıldığında, *R. flavafaciens* popülasyonunun düşük olduğu (P<0,01) bulunmuştur. Sonuçlar, probiyotik karışımının hem mikrobiyal metabolik aktiviteyi hem de ruminal mikroorganizma popülasyonunu değiştirebileceğini göstermiştir.

Anahtar sözcükler: Kısa zincirli yağ asitleri, nutrigenomikler, probiyotikler, ruminomikler, selülolitik bakteriler.

Introduction

The "omic" technologies that are used in an extensive range of an execution area, are scoped at the determination of genes (genomics), proteins (proteomics), metabolites (metabolomics), nutritional genomics (nutrigenomica) and rumen microbial genomics (RuminOmics project) (8). Rumen environment consists of a variety of microorganisms, like cellulolytic, proteolytic, amylolytic and so on, which act in harmony to degrade several nutrients to contribute energy and protein metabolism of the host. Gut microbial communities (microbiomes) through genomics and metagenomics and

its underlying functions through the construction of a gene catalogue can improve ruminal microorganism actions and rumen health as well as metabolic health of host by modificating the fermentation parameters (15). The digestibility of nutrients is significant for ruminal fermentation to produce SCFA which is the main source of energy for bacteria and host. Cellulolytic bacteria, such as *Ruminococcus albus* and *Ruminococcus flavafaciens* utilize the cellulose by excreting exo-1,4-β- glucanase, endo-1,4-β-glucanase and cellodextrinase 1,4-β-glucosidase (10) and rapidly proliferate when ammonia concentration is adequate. However, with the reduction of the rumen pH, forage digestibility is reduced incrementally due to the decreased cellulolytic bacteria population (17). Because rumen pH is an important determinant of rumen cellulolytic bacteria activity, as they are negatively affected by the reduction of pH below 6.00 (24). For this purpose, to improve the ruminal flora and/or fauna, some biotechnological feed additives were developed, such as probiotics, that are considered as an alternative to antibiotics. Probiotics can be defined as viable microorganisms that have a beneficial effect on the health and performance of the host (23). Chaucheyras-Durand and Durand (4) reviewed that the most significant effects of probiotics have been reported when they have been included in the diet of animals for the gut microbiota and the animal, stabilizing the rumen pH and promoting the rumen health by reducing risk of acidosis. We hypothesized that this probiotics blend may exert an additive effect on rumen fermentation parameters and *R. albus* and *R. flavefaciens* abundance to help the cellulose degradation. The purpose of this study was to evaluate the effects of probiotics blend addition on ruminal fermentation, and ruminal microbiome genomics by the use of real-time PCR for monitoring cellulolytic bacterial species in the artificial rumen of cattle and to indicate the precise population level of *R. albus* and *R. flavefaciens* by quantifying the gene of each species.

Materials and Methods

Experimental diets and procedure: To stimulate in vitro rumen fermentation, dried alfalfa hay was used as a substrate in fermenters for HFT method (14) during the experiment. The alfalfa hay was provided by a commercial farm**.** Nutritional composition of alfalfa were analyzed according to Association of Official Analytical Chemists (1), and presented in Table 1. The probiotics were obtained from a commercial company. The probiotics blend was comprised of *Saccharomyces cerevisiae, Bacillus subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Streptococcs thermophiles, Lactobacillus bulgaricus* strains along with the distillated water, molasses of organic sugar cane, salt, calcium oxide, and magnesium oxide.

Table 1. Chemical composition of alfalfa

Chemical composition	
Dry matter, %	93.00
Crude protein, %	9.51
Ash, %	8.20
Crude fiber, %	32.00
Acid detergent fiber, %	46.00
Neutral detergent fiber, %	65.82
Ether extract, %	1.10
ME, kcal/kg	1510

In vitro fermentation technique: Fermentation was performed according to a modified HFT (14). Two hundred milligrams of the dried alfalfa substrate were incubated with 30 ml of a ruminal buffered suspension with 0,1 µl /ml probiotics (PRO) to reach the 1×10^{10} cfu/g of probiotic blend or without an additive as a control (CON) at 39 °C. Buffer suspension consisted of 20 ml buffer solution and 10 ml rumen fluid. Rumen fluid was obtained from a slaughtered cattle at a local slaughterhouse. The owner indicated that these bulls were fed a diet consisting of concentrate and straw (0.79:0.21). Rumen fluid was immediately transferred to preheated thermos and brought to the laboratory to start the fermentation. Then, rumen fluid immediately mixed and strained into syringes which were previously heated in an incubator (39 °C) and filled with the buffer solution (Macro Element Solution: Na_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$; Micro Element Solution: $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $FeCl_3.6H_2O$; Buffer Solution=$NaHCO_3$, NH_4HCO_3; Resazurin Solution=Resazurin; Reductant Solution= $Na_2S.7H_2O$, NaOH) which was bubbled with CO_2. The syringes were placed into an incubator for 3, 6, 12, 24 and 48 hours and kept at 39 °C. A total of 40 syringes were used during the experiment.

Rumen sampling and analysis: After each incubation, the rumen fluid samples were collected from 4 syringes of each group and were strained into the individual beakers with a sterile cheesecloth to stop the fermentation. Each rumen fluid sample was divided into 4 portions. The first portion of each sample was transferred into a 10 ml tube for pH measurement. The pH was measured immediately with a pH-meter (Hanna Instruments). The second portion was transferred into a 5 ml tubes and then the tubes were stored at −20°C for the ammonia nitrogen measurement. The third and the last portions were transferred into the 5 ml vials and stored at −20 °C for the SCFA and the molecular analysis, respectively. Finally, 0.1 ml rumen fluid sample was taken into the tubes and suspended with 0.9 ml formaldehyde (37%) to determine total bacteria count. The number of bacteria were determined using a phase-contrast

microscope with Thoma bacteria counting chamber (depth: 0.02 mm, small square area: 0.0025 mm^2). For the determination of total protozoan number in fermented fluids, 1 ml rumen samples were taken and supplied with 1 ml of protozoan counting solution (0.6 g methyl green, 8 g NaCl, 100 ml 37% formaldehyde). The protozoan numbers were counted with the Fuchs-Rosenthal counting chamber (depth: 0.2 mm, small square area: 0.0625 mm^2) using a light microscope (3, 7).

Ammonia nitrogen in rumen fluid was measured using spectrophotometry by using indophenol blue method at 546 nm according to the method described by Chaney and Marbach (2). Concentration of SCFA were determined by gas chromatography according to Geissler et al (6). Briefly, frozen rumen samples were thawed at 4°C and rumen fluids were centrifuged at 4,000 rpm for 15 min at 4°C. One ml of supernatant was then transferred to an Eppendorf tube and mixed with 0.2 mL ice-cold 25% metaphosphoric acid solution. Then, tubes were placed in an ice bath for 30 min. Subsequently, these tubes were centrifuged again at 11,000 rpm for 10 min at 4°C and the supernatant was transferred into gas chromatography vials to determine acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid concentrations. Samples were analyzed by using gas chromatography (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with a capillary column (TR-151035, TRB-FFAP, 30 m x 0.53 mm). The column temperature was programmed to increase gradually from 120ºC to 160ºC during the analysis. In addition, the injector port and flame ionization detector (FID) temperatures were fixed at 230ºC and 250ºC, respectively. The injection volume was set to 1 μL and analyses were performed in duplicate. Total protozoa and bacteria were counted using counting chambers (0.1 mm and 0.02 mm depths, respectively) under a microscope.

DNA extraction and real-time PCR: Rumen fluid samples were stored without any preservative at −20°C for molecular analysis. Genomic DNA extraction was performed using the easy DNA Kit (Thermo Scientific) from *R. albus* and *R. flavafaciens* according to the manufacturers' recommendations. Quantitative real-time PCR was performed using previously published primer sets as shown in Table 2. All primers were obtained from Novagentek Laboratory Products and Technologies Ltd. Co. (Ankara, Turkey). The quantification of *R. albus* and

R. flavafaciens DNA in total rumen DNA was carried out using Rotor-GeneQ (Qiagen, Germany). The reaction mixture (25 μL) consisted of 12.5 μL of Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics, Indianapolis, IN, USA), 0.2 μmol/L of each primer set and 5 μL of the template DNA. The real-time PCR conditions involved one cycle at 95°C for 5 min for initial denaturation and then 45 cycles of 95°C for 20 s followed by 60°C for 50 s. Detection of the fluorescent product was set at the last step of each cycle. Prior to cloning, PCR products were amplified by using the reaction mix containing 75 mM Tris–HCl (pH 8.8), 20 mM $NH_4(SO_4)2$, 2.4 mM $MgCl_2$, 10 pmol of each primer, 0.2 mM dNTP, and 5 U of Taq DNA polymerase (Thermo Scientific Company) and 3 μl of DNA. The mixture for PCR was preheated at 94 °C for 5 min, subjected to 30 cycles of 1 min at 94 °C, 1 min at the required temperature for each primer pair (Table 2), 2 min at 72 °C and a final 7 min incubation at 72 °C. One positive amplicon from each species (*R. albus and R. flavafeciens*) was ligated into the Pjet 1.2 cloning vector (Thermo Scientific Company) and transformed into competent *E. Coli* DH5α cells. Then, the clones were tested for correct insertion size by colony PCR amplification with Pjet1.2 forward and reverse primers supplied by the manufacturer. The positive amplicons quantitated spectrophotometrically and used as standards in 10-fold dilutions in the qPCR assay, carried out using QuantiTect Probe RT-PCR kit (Qiagen, Germany) in a Rotor-GeneQ (Qiagen, Germany).

Statistical analysis: All data were analyzed using MIXED procedure of SPSS (V22.0; SPSS Inc., Chicago, IL, USA). The effect of group, sampling time and their interaction on pH, NH3, acetic, propionic, isobutyric, butyric, isovaleric, valeric, total protozoa and bacteria, a/p ratio, *R. albus* and R. *flavafeciens* were analyzed by using following model with repeated measures:

Where dependent variable; overall mean; effect of the group (= Group 1 and Group 2); effect of sampling time (= 1, 2, 3, 4 and 5); interaction between group and sampling time; and residual error. Group, sampling time and their interaction were assessed as a fixed effect. P≤0.05 was considered as significant in all analyses. When a significant difference was revealed, any significant terms were compared by simple effect analysis with Bonferroni adjustment.

Table 2. Oligonucleotide primers used for qPCR in rumen fluid

Target bacteria	Item	Primer sequence (5'-3')	Size	References
R. albus	Forward	*CCCTAAAAGCAGTCTTAGTTCG*	175 bp	Koike and Kobayashi (22)
	Reverse	*CCTCCTTGCGGTTAGAACA*		
R. flavafeciens	Forward	*GGACGATAATGACGGTACTT*	835 bp	Tajima et al. (23)
	Reverse	*GCAATC(CT)GAACTGGGACAAT*		

Results

No significant interaction effect on ruminal pH and ammonia-N concentration was observed between the treatment and incubation time (Table 3). As expected, NH$_3$-N, acetate, propionate, butyrate, *iso*butyrate, valerate, *iso*valerate, and total SCFA concentrations were increased with the 3, 6, 12, 24 and 48 h of incubation (P < 0.001). On the other hand, supplemental probiotics tended to increase the total SCFA concentration (from 38.17 to 94.21 mM/l vs. from 37.77 to 73.25mM/l) in comparison to non-supplemented control fermenter fluids, however, interaction between the groups were not significant (P = 0.063) (Table 3). A significant interaction effect was observed in butyrate (P = 0.042), valerate (P = 0.045) and *iso*valerate (P = 0.028) concentrations between treatment groups and incubation times (Table 4).

Table 3. Effects of probiotics on pH value, NH$_3$-N (mmol/l), total protozoa and bacteria (log$_{10}$ cfu/ml) number (from 3 to 48 h of incubation) in fermenter fluids during the incubation time.

| Parameters	Group	Time					SEM	Mean effect (Group)	P		
		3	6	12	24	48			G	T	GxT
pH	CON	6.903	6.913	6.860	6.930	6.937	0.079	6.909 ± 0.035			
	PRO	6.925	6.802	6.712	6.815	6.990		6.849 ± 0.035	0.243	0.258	0.630
Mean effect (time)		6.914	6.858	6.786	6.873	6.964	0.056				
NH3	CON	11.845	13.138	19.515	29.033	36.745	1.818	22.055 ± 1.289			
	PRO	10.850	12.425	18.730	28.165	35.108		21.056 ± 1.289	0.279	<0.001	0.998
Est Mean effect (time)		11.348^d	12.781^d	19.123^c	28.599^b	35.926^a	1.509				
Total protozoa	CON	5.92^a	5.772$^{b.\ B}$	5.7$^{b.\ B}$	5.492$^{c.\ B}$	5.183$^{d.\ B}$	0.020	5.613 ± 0.009			
	PRO	5.89^a	5.94$^{a.\ A}$	5.885$^{a.\ A}$	5.667$^{b.\ A}$	5.407$^{c.\ A}$		5.758 ± 0.009	<0.001	<0.001	<0.001
Mean effect (time)		5.905	5.856	5.792	5.580	5.295	0.014				
Total bacteria	CON	8.468	8.492	8.407	8.400	8.425	0.047	8.439 ± 0.030^B			
	PRO	8.497	8.605	8.500	8.487	8.482		8.514 ± 0.030^A	0.007	0.096	0.869
Mean effect (time)		8.482	8.549	8.454	8.444	8.454	0.037				
Total SCFA	CON	37.765	48.070	69.277	81.700	73.245	4.639	62.012 ± 2.075			
	PRO	38.170	50.390	71.165	74.535	94.212		65.694 ± 2.075	0.219	<0.001	0.063
Mean effect (time)		37.967^c	49.23^c	70.221^b	78.117^{ab}	83.729^a	3.281				

$^{a-d}$ or $^{A-B}$: Means in the same row or column followed by different superscripts differ significantly (P <0.05); CON= Control group that is without any supplementation; PRO= Treatment group with probiotic addition, 1 x 10^{10} cfu/g; G= Effect of the trial groups; T= Effect of the relative to incubation time; G x T= The experimental groups by incubation time interaction

Table 4. Effects of probiotics SCFA (mM/l) concentration (from 3 to 48 h of incubation) in fermenter fluids during the incubation time.

Parameters	Group	Time					SEM	Mean effect (Group)	P		
		3	6	12	24	48			G	T	GxT
Asetic a.	CON	24.117	31.112	43.825	50.845	45.610	1.818	39.102 ± 6.561			
	PRO	24.262	32.630	45.297	46.230	58.387		41.361 ± 6.561	0.234	<0.001	0.076
Mean effect (time)		24.19^b	31.871^b	44.561^a	48.537^a	51.999^a	6.756				
Propionic a.	CON	6.337	8.300	12.345	14.310	12.628	1.009	10.784 ± 0.699			
	PRO	6.400	8.605	12.477	13.105	16.368		11.391 ± 0.699	0.247	<0.001	0.057
Mean effect (time)		6.396^b	8.452^b	12.411^a	13.707^a	14.498^a	0.829				
Isobytüric a.	CON	0.550	0.650	0.965	1.355	1.348	0.065	0.974 ± 0.029			
	PRO	0.562	0.672	1.017	1.300	1.680		1.047 ± 0.029	0.086	<0.001	0.051
Mean effect (time)		0.556^c	0.661^c	0.991^b	1.328^a	1.514^a	0.046				
Byturic a.	CON	4.962^b	5.96^b	9.195^a	10.887^a	9.382$^{a.\ B}$	1.261	8.077 ± 1.140			
	PRO	5.09^c	6.352^c	9.247^b	9.912^{ab}	12.262$^{a.\ A}$		8.573 ± 1.140	0.203	<0.001	0.042
Mean effect (time)		5.026	6.156	9.221	10.400	10.822	1.186				
Isovaleric a.	CON	0.91^c	1.073^{bc}	1.685^b	2.615^a	2.775$^{a.\ B}$	0.153	1.812 ± 0.068			
	PRO	0.94^d	1.12^d	1.783^c	2.46^b	3.61$^{a.\ A}$		1.982 ± 0.068	0.087	<0.001	0.028
Mean effect (time)		0.925	1.096	1.734	2.538	3.192	0.108				
Valeric a.	CON	0.89^d	0.973^{cd}	1.267^{bc}	1.683^a	1.505$^{ab.\ B}$	0.085	1.263 ± 0.038			
	PRO	0.915^d	1.01^{cd}	1.342^{bc}	1.527^b	1.905$^{a.\ A}$		1.340 ± 0.038	0.167	<0.001	0.045
Mean effect (time)		0.902	0.991	1.305	1.605	1.705	0.060				
a/p	CON	3.806	3.762	3.551	3.548	3.607	0.077	3.655 ± 0.068			
	PRO	3.791	3.795	3.635	3.527	3.568		3.663 ± 0.068	0.738	<0.001	0.520
Mean effect (time)		3.799^a	3.779^a	3.593^b	3.537^b	3.588^b	0.072				

$^{a-d}$ or $^{A-B}$: Means in the same row or column followed by different superscripts differ significantly (P <0.05); CON= Control group that is without any supplementation; PRO= Treatment group with probiotic addition, 1 x 10^{10} cfu/g; G= Effect of the trial groups; T= Effect of the relative to incubation time; G x T= The experimental groups by incubation time interaction.
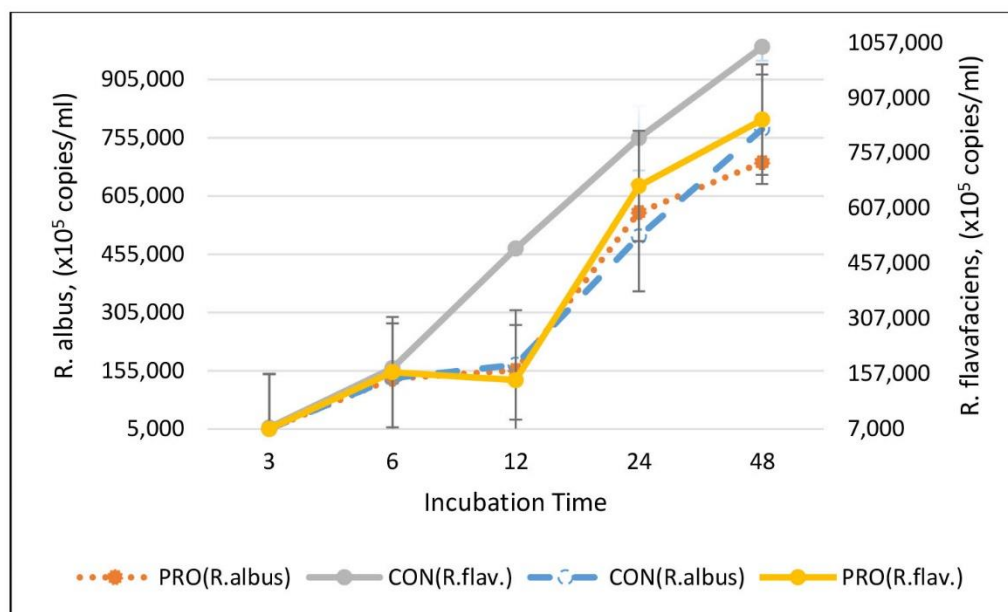
Figure 1. Effect of group (Control; CON, Probiotics; PRO) by time interaction on R. *albus* and R. *flavafaciens* population in fermenter fluid.

A significant interaction effect was observed in the total protozoa number (P < 0.001). Total protozoa (4.45 %) and total bacteria numbers (0.60%) were found to be higher in probiotic supplemented fluid than in non-supplemented control fluid at the last incubation time (Table 3). As expected, numbers of both *R. albus* and *R. flavafaciens* were increased with the advanced incubation time. Dietary supplementation of the probiotic cocktails to the fermenters did not influence the number of copies of *R. albus*. However, population of *R. flavafaciens* was found to be lower (P = 0.009) in the probiotics supplemented group as compared with non-supplemented control group. No significant interaction effect on cellulolytic bacteria numbers was observed between the treatment groups and incubation time (Figure 1).

Discussion and Conclusion

Bacterial and fungal probiotics confer beneficial effects on a rumen environment by improving the development of rumen milieu and stimulating the maintenance of a stable fermentation. Ruminant nutrition experiments are usually accompanied by the measurement of number of parameters, such as rumen pH, SCFA and ammonia concentration to find out the effects of dietary treatments on a host animal (5). It is generally known that these parameters are directly related to the rumen microorganisms which are influenced by the dietary substrates and bioactive compounds. Similar to the previous study designs, the present study aimed to investigate the influence of a probiotics blend on rumen fermentation and ruminal bacteria abundance by using an *in vitro* model.

Rumen pH, NH_3-N and SCFA concentrations, which are directly related to ruminal fermentation, are important factors for rumen function, flora, and fauna. Over the last decade, a considerable number of studies has reported that the probiotics, mostly of yeasts, to modify the ruminal fermentation by stabilizing pH and regulating the SCFA absorption (4, 22). Probiotics affects used for modifying the rumen fermentation was considered positive in terms of energy efficiency when the SCFA concentration change because the volatile fatty acid meets most of the daily energy requirement for ruminants (22). In our experiment, an addition of the probiotics blend with substrate at different incubation times did not alter the pH value. On the contrary, NH_3-N and SCFA concentrations were influenced by the incubation time. The effect of probiotics on rumen fermentation as well as SCFA production were generally species-dependent. In our experiment the probiotic blend including yeast was used and it is considered the different species might be effective in different incubation time. Regardless of the incubation time, supplementation of probiotics did not affect rumen pH, NH_3-N and SCFA concentrations. This is consistent with Yang et al. (25) who found no differences in fermenter pH, total ruminal SCFA concentrations, and their molar proportions between the control and the direct fed microbial supplemented group. Moreover, Zhong et al. (26) revealed that inoculation of fresh rumen liquid as a probiotic supplement had no significant effect on ruminal pH, ammonia-N and total SCFA concentrations in lambs. Whereas, Pinloche et al. (20) reported that dietary yeast supplementation increased the pH value and total SCFA level, decreased the NH_3 concentration in rumen with the

increasing levels of *S. cerevisiae* from 0.5 g/d to 5 g/d. However, Paengkoum et al. (18) concluded that the combined use of *S. cerevisiae* with *Lactobacillus acidophilus* has adverse effect on rumen pH value, SCFA and NH_3 concentrations. However, previous study results are contradictory, mostly due to the dose and type of the probiotic supplements that are used in the experiments. It is known that the pH value is influenced by rumen SCFA concentration which is also the major precursor for subacute ruminal acidosis (SARA) (9). Khafipour et al. (9) demonstrated a clear relationship between subacute ruminal acidosis and particular rumen bacterial population that might be protective. Thereby, even in the existence of numerically higher total SCFA concentration (non-supplemented fluid; 73.25 vs probiotic supplemented group 94.21), an unchanged pH in the current study may be an important finding for the prevention of SARA. However, more in vivo animal studies and/or SARA challenged studies are needed to prove the effect of probiotics on rumen fermentation characteristics, performance, and health of the host animal.

Changes in rumen microbiota and protozoa abundance are of great significant for rumen fermentation characteristics and the health of the host. These parameters are directly influenced by diet and as well as by feed additives such as probiotics. According to our results, supplementation of the probiotic blend to in vitro fermenters significantly increased total protozoa and bacteria counts in rumen fluid. Similarly, Newbold et al. (16) concluded that *S. cerevisiae* significantly influenced rumen total bacteria in an in vitro fermenter (Rusitec). As expected, our results showed that *R. albus* and *R. flavafaciens* were influenced by the incubation time. However, no significant interaction was observed between the group and the incubation time. Contrary to our expectation, number of *R. flavafaciens* in rumen fluid were found to be lower than control group. These findings are consistent with the study of Mathieu et al. (13) which reported that the yeasts reduced the cellulolytic bacteria population in fistulated sheep while inconsistent with previous studies (12, 19) which concluded that probiotics could have beneficial effect on rumen bacterial populations by promoting the growth of cellulolytic bacteria. Newbold et al. (16) revealed that *S. cerevisiae* stimulated the cellulolytic bacteria count in Rusitec. However, most of the earlier work focused on the effects of *S. cerevisiae* on rumen fermentation parameters and bacteria population rather than probiotics blend. Discrepancies between our results and previous studies is probably due to the differences in the strains of probiotics, doses of the probiotic blend or the experimental design. On the other hand, the growth rate of R. *flavefaciens,* but not R. *albus*, was lowered by less than 0.03 mmol/L of nitrate in rumen fluid (11). In present study, the nitrate level of the rumen samples did not analyzed but it might be higher than that level regarding with the strains of probiotics. Because in *Bifidobacterium spp.* species that was used in present probiotics blend, the nitrate reduction is negative (21).

Our results showed that the probiotics blend might modulate both microbial metabolic activity and population of the ruminal microbes. However, more research needs to be conducted to determine their effects on rumen fermentation characteristics and bacterial population with an in vitro and in vivo experiments design.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declare that they have no conflict of interest.

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