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Ruminants

In vitro study of postbiotics from *Lactobacillus plantarum* RG14 on rumen fermentation and microbial population

Wan Ibrahim Izuddin[1], Teck Chwen Loh[1,2*], Anjas Asmara Samsudin[1,2], Hooi Ling Foo[3,4]

[1] *Universiti Putra Malaysia, Faculty of Agriculture, Department of Animal Science, Serdang, Selangor, Malaysia.*
[2] *Universiti Putra Malaysia, Institute of Tropical Agriculture and Food Security, Serdang, Selangor, Malaysia.*
[3] *Universiti Putra Malaysia, Faculty of Biotechnology and Biomolecular Sciences, Department of Bioprocess Technology, Serdang, Selangor, Malaysia.*
[4] *Universiti Putra Malaysia, Institute of Bioscience, Serdang, Selangor, Malaysia.*

ABSTRACT - An *in vitro* study was carried out to identify the effects of different inclusion levels of postbiotics from *Lactobacillus plantarum* RG14 on rumen fermentation profiles, gas production kinetics, and microbial population in rumen fluid collected from goats. Postbiotics were added at different levels (0, 0.3, 0.6, 0.9, and 1.2%) and incubated for 72 h with 200 mg dry matter (DM) of a substrate containing 60% Guinea grass and 40% commercial concentrate. The experiment was conducted in triplicate on different days with four replications per treatment. Rumen fermentation kinetics, pH, organic matter digestibility (OMD), volatile fatty acids (VFA), and microbial populations were investigated. Net gas production and gas production from the immediate soluble fraction (a) increased linearly, and the volume of gas produced from the insoluble fraction (b) and potential gas production (a + b) quadratically increased with the increasing levels of postbiotics. A significant linear increase in OMD was observed for increasing postbiotic levels. Total and individual molarity of ruminal VFA and acetic acid to propionic ratio were also significantly increased by postbiotic inclusion. Populations of total bacteria, cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*), and total protozoa were significantly increased in the postbiotic treatment. Postbiotics in the ruminal fluid *in vitro* enhance rumen fermentation and improve digestibility and VFA production without any adverse effects on pH.

Key Words: feed additive, fermentation profile, *Lactobacillus*, ruminal kinetics, rumen microorganisms, volatile fatty acids

Introduction

The development of antibiotic-resistant bacteria due to the excessive use of antibiotics in animal production poses significant health risks to both livestock and humans (Van Boeckel et al., 2015). In ruminants, the use of antibiotics to manipulate the microbial ecosystem to improve fibrous feed use has been discouraged due to several disadvantages such as toxicity, potential allergies, and residues in livestock products (Ramaswami et al., 2005). Direct-fed microbials (DFM) or probiotics, which are defined as "a source of live naturally-occurring microorganisms", are currently being used as supplements for ruminants (Yoon and Stern, 1995). Lactic acid bacteria as probiotics have been used in ruminant diets to enhance the beneficial microflora population, which in turn will prevent pathogenic microbial establishment. They positively influence the microbial ecosystem by establishing native gastrointestinal microbes in young calves and by contributing to the equilibrium of microbial groups in the gastrointestinal system (Bae et al., 2003; Ramaswami et al., 2005). Although the beneficial effects of probiotics are undisputed, they could represent a threat to the host. Some probiotics have antibiotic resistance genes, particularly plasmid-encoded bacteria, which can be transferred between organisms (Marteau and Shanahan, 2003).

In this context, postbiotic produced from lactic acid bacteria contain no living cells and are a potential feed additive in livestock and poultry production (Thu et al., 2011a). Postbiotics contain intermediates and/or final products in metabolism of *Lactobacillus* sp., mainly lactic and acetic acid and antimicrobial peptides known as bacteriocins (Foo et al., 2005; Thanh et al., 2010; Thu et al., 2011a; Choe et al., 2013). Postbiotics exhibit probiotic effects that reduce the pH in the intestines, increase the lactic acid bacteria population, and decrease the *Enterobacteriaceae* population (Foo et al., 2005). They also improve growth performance, increase intestinal villus

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***Corresponding author:** tcloh@upm.edu.my

height, and decrease faecal *Enterobacteriaceae* counts in broilers (Loh et al., 2010), laying hens (Choe et al., 2012), and piglets (Thu et al., 2011b).

The use of postbiotics in monogastric farm animals is well established; however, information about the use of postbiotics in ruminant diets is limited. In ruminants, direct-fed microbes such as *Lactobacilli* have been used as feed additives in improving animal welfare and performance. The interaction of lactic acid bacteria with rumen microorganisms to enhance rumen fermentation and inhibition of harmful microorganisms due to the presence of antimicrobial substances such as bacteriocins are proposed by Weinberg et al. (2003) as the mode of action of lactic acid bacteria in the rumen. Lactic acid-producing bacteria produce a steady lactic acid in the rumen, assists the microflora to accustom with the lactic acid accumulation, enhance lactate-utilizing bacteria, and stabilize ruminal pH (Yoon and Stern, 1995; Seo et al., 2010; Retta, 2016). Similar effect could be achieved by supplementing postbiotics as a feed additive. We therefore evaluated the effects of postbiotics produced by *Lactobacillus plantarum* (*L. plantarum*) on rumen fermentation and microbial population *in vitro*.

Material and Methods

This study was conducted in Serdang, Selangor, Malaysia (2°58'58" N and 101°44'06" E), following guidelines of the university research policy.

The postbiotics were produced by *Lactobacillus plantarum* RG14. The stock culture was revived twice in de-Mann Rogosa Sharpe (MRS) broth by incubating at 30 °C for 48 and 24 h, respectively. The streak plate was conducted on MRS agar and incubated for 48 h at 30 °C; subsequently, a single colony was picked and incubated in MRS broth for 48 h. The colony was then subcultured in MRS broth, incubated for 24 h at 30 °C, and used as inoculum. One percent of the culture was inoculated into reconstituted media and incubated for 24 h at 30 °C. Centrifugation was performed at 10,000 × *g* to separate bacterial cells, and the postbiotics were harvested by collecting the supernatant. The collected postbiotics were examined for inhibitory activity by conducting an agar well diffusion assay, using *Pediococcus acidilactici* as the indicator organism.

Two male goats (30±2 kg body weight), fitted with a permanent rumen cannula, were housed individually in raised floor pens. Fresh water and mineral blocks were provided *ad libitum*. The goats were maintained on a daily diet containing 60% dry matter (DM) fresh Guinea grass (*Panicum maximum*) with 40% DM commercial concentrate and fed twice daily at 08:00 and 17:00 h. Rumen fluid was taken in the morning before feeding and transferred immediately into pre-warmed (39 °C) insulated vacuum flasks to ensure anaerobic conditions. In the laboratory, rumen fluid was continuously flushed with CO_2 to maintain the anaerobic condition. The collected rumen fluids samples were pooled, filtered through four layers of cheesecloth into a pre-warmed conical flask bath, mixed with buffer at the ratio of 1:2, and kept at 39 °C in a water bath prior to transfer to fermentation syringes. The buffer solution was prepared according to Menke and Steingass (1988).

In vitro gas production was evaluated according to Menke and Steingass (1988). Guinea grass and commercial concentrate were used as a substrate and ground to pass through a 1.0-mm sieve. The combination of Guinea grass and commercial concentrate were used as the substrate for *in vitro* rumen fermentation (Table 1). The substrate was weighed to 0.2±0.0001 g DM to contain 0.12 g of DM of Guinea grass and 0.08 g of DM of concentrate and dispensed into 100-mL calibrated glass syringes fitted with pistons. Approximately 0.2±0.0001 g DM of standard hay and standard concentrate (University of Hohenheim, Stuttgart, Germany) with known gas production were also used for incubation (Menke and Steingass, 1988). Subsequently, 30 mL of buffered rumen fluid were dispensed into syringes containing substrate with different levels of postbiotics, syringes with postbiotics without substrate, syringes containing hay and concentrate standards, and blank syringes without substrate. Postbiotic inclusion levels were 0 (control), 0.3, 0.6, 0.9, and 1.2%, based on the rumen fluid media. The syringes were incubated in a water bath at a constant temperature of 39±0.1 °C. Gas production was recorded at 3, 6, 12, 24, 48, and 72 h of incubation. The *in vitro* experiment was conducted in three runs on separate days, with four replications per treatment.

At the end of the incubation, the pH of the rumen fluid was measured immediately using a pH meter (Mettler-Toledo Ltd., England, UK). An aliquot of 2 mL of the rumen

Table 1 - Chemical composition (g kg^{-1} DM) of Guinea grass and commercial concentrate

	Guinea grass	Concentrate[1]	Combination[2]
Chemical composition (g kg^{-1} DM)			
Dry matter (DM)	924.34	867.73	906.32
Ash	110.50	70.50	95.33
Crude protein	115.40	122.10	118.80
Ether extract	47.68	25.50	29.18
Neutral detergent fibre	771.33	385.67	729.33
Acid detergent fibre	478.33	191.67	358.67
Lignin	266.00	91.67	201.33

[1] Commercial goat concentrate.
[2] Combination of 60% Guinea grass and 40% commercial concentrate.

fluid was collected for microbial population determination and another 10 mL of the fluid were taken for volatile fatty acids (VFA) analysis. The sample was then kept in a freezer at −20 °C until analysis. The VFA of the rumen fluid were determined using a 6890N Network GC System gas chromatograph (Agilent Technologies) according to Filípek and Dvořák (2009). The rumen fluid was acidified with 25% metaphosphoric acid in the ratio of 4:1 (v/v) and centrifuged at 3,000 × *g* for 10 min. The supernatant was collected, filtered, and used for VFA determination. As internal standard, 4-methyl-n-valeric acid (Sigma, St. Louis, MO) was used.

Quantification of the microbial population in the rumen fluid was performed after 72 h of incubation. Extraction of DNA from the ruminal fluid was carried out using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The concentration of the extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington. DE). The populations of total bacteria, *L. plantarum*, *Ruminococcus albus* (*R. albus*), *Ruminococcus flavefaciens* (*R. flavefaciens*), *Fibrobacter succinogenes* (*F. succinogenes*), and protozoa were analysed by using qPCR. Absolute quantification of microbes in the rumen fluid was performed based on the standard curve of amplification of target microbes. Approximately 20 µL of qPCR master mix were prepared for each reaction, using the QuantiNova™ SYBR Green PCR kit (Qiagen, Hilden, Germany), which consists of 10 µL of 2X SYBR Green Master Mix, 1 µL of 14 µM forward primer, 1 µL of 14 µM reverse primer, 2 µL of DNA samples, and 6 µL of RNase-free water. Real-time qPCR (Table 2) was performed with the Bio-Rad CFX96 Real-time PCR system (Bio-Rad Laboratories, Hercules, CA), using optical grade plates. The qPCR cycling conditions consisted of an initial heat activation at 95 ºC for 2 min, followed by 40 cycles of denaturation at 95 ºC for 5 s and a combined annealing and extension temperature, depending on the target microbes. Melting curve analysis was performed at the end of the amplification cycle to confirm the specificity of amplification.

Standard hay and standard concentrate (University of Hohenheim, Stuttgart, Germany), with an average gas production of 49.61 and 61.13 mL, respectively, were used for calibration. Blank (buffered ruminal fluid only) and postbiotics only (buffered rumen fluid and postbiotics) were used to correct the gas production in the fermentation of the substrate by estimating gas production based on rumen fluid and postbiotics. A blank was also used to estimate the DM in the rumen fluid and to correct the DM degradation of the substrate. Gas production data were fitted into the model as described by Ørskov and McDonald, (1979): $Y = a + b\ (1 - e^{-ct})$, in which Y = volume of gas produced at time t (mL), a = volume of gas produced from the immediately soluble fraction (mL), c = gas production rate constant from the insoluble fraction (mL/h), and t = incubation time. Organic matter digestibility (OMD) of the substrate was determined following the equation of Menke et al. (1979): OMD (%) = 14.88 + 0.889GP + 0.45CP + 0:0651AC, in which GP = net gas produced at 24 h (mL/200 mg) of incubation, CP = percentage of crude protein content, and AC = percentage of ash content of the substrate.

The experiment was conducted as a completely randomized design according to the following statistical model:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

in which, Y_{ij} = observation of the effect of different postbiotic levels, µ = overall mean of the observation, α_i = effect of different levels of postbiotic (i = 0, 0.3, 0.6, 0.9, 1.2) in *in vitro* rumen fermentation, and ε_{ij} = random error.

Table 2 - Sequence of polymerase chain reaction primers used to target total bacteria, *L. plantarum*, *F. succinogenes*, *R. albus*, *R. flavefaciens*, and total protozoa

Target microbe	Primer	Annealing temperature (°C)	References
Total bacteria	F - CGGCAACGAGCGCAACCC R - CCATTGTAGCACGTGTGTTAGCC	55	(Samsudin et al., 2014)
L. plantarum	F - CTCTGGTATTGATTGGTGCTTGCAT R - GTTCGCCACTCACTCAAATGTAAA	60	(Matsuda et al., 2009)
F. succinogenes	F - GTTCGGAATTACTGGGCGTAA A R - CGCCTGCCCCTGAACTATC	55	(Lane, 1991)
R. albus	F - CCCTAAAAGCAGTCTTAGTTCG R - CCTCCTTGCGGTTAGAACA	55	(Koike and Kobayashi, 2001)
R. flavefaciens	F - TCTGGAAACGGATGGTA R - CCTTTAAGACAGGAGTTTACAA	60	(Koike and Kobayashi, 2001)
Total protozoa	F - CTTGCCCTCYAATCGTWCT R - GCTTTCGWTGGTAGTGTATT	55	(Sylvester et al., 2004)

F - forward; R - reverse.

The data were subjected to one-way ANOVA, using the General Linear Model (GLM) of the software package SAS (Statistical Analysis System, version 9.2). Duncan's Multiple Range Test was used to identify significant differences between the treatments. Orthogonal polynomial contrasts were used to determine the linear and quadratic effects of increasing postbiotic levels. Differences between treatment means were considered significant at P<0.05.

Results

A significant linear increase (P<0.05) in net gas production at all incubation times was observed for increasing postbiotic levels (Table 3). Gas production from the fermentation of the insoluble fraction (b) and potential extent of the gas production (a + b) significantly differed between the different inclusion levels and the control. Gas production from the fermentation of the soluble fraction (a) was significantly higher (P<0.05), starting at 0.6% of postbiotic inclusion compared with the control. The OMD also showed a significant linear increase (P<0.05) with increasing inclusion levels.

The pH was not affected (P>0.05) by the inclusion of postbiotics. In contrast, acetic, propionic, butyric, and total VFA linearly increased (P<0.05) with increasing inclusion levels (Table 4). The propionic acid and total VFA significantly different (P<0.05) at all inclusion levels of postbiotics. The ratio of acetic to propionic acid was not affected (P>0.05).

A significant quadratic increase (quadratic; P<0.05) was observed in the total bacteria population during incubation with the addition of postbiotics (Table 5). However, the *L. plantarum* population was not affected (P<0.05) by the different inclusion levels. Interestingly, the populations of three major fibrolytic bacteria, namely *F. succinogenes*, *R. albus*, and *R. flavefacien*s, linearly increased (P<0.05) with increasing inclusion levels. A significant quadratic increase (quadratic; P<0.05) in total protozoa was observed, starting from a postbiotic level of 0.6%.

Table 3 - Effect of different levels of postbiotic inclusion on gas production at 72 h of *in vitro* incubation

Parameter	Level of postbiotic inclusion (%)					SEM	Contrast, P-values	
	0 (control)	0.3	0.6	0.9	1.2		Linear	Quadratic
Net gas production (mL/200 mg DM)								
Incubation time (h)								
3	7.21c	8.42c	9.78b	10.60ab	11.30a	0.39	<0.0001	0.5959
6	14.00d	15.00c	16.80b	17.80a	17.90a	0.24	<0.0001	0.0045
12	25.50c	26.40cb	27.80ab	28.70a	29.20a	0.49	<0.0001	0.4294
24	37.90c	39.00c	40.60b	41.90ab	42.10a	0.48	<0.0001	0.1511
48	75.00c	77.50b	79.10ab	80.40a	80.50a	0.62	0.0003	0.0457
72	79.10b	82.30a	83.00a	84.10a	84.30a	0.62	<0.0001	0.0143
Gas production parameter								
a (mL)	−6.06c	−5.54c	−4.38b	−3.16a	−3.68a	0.30	<0.0001	0.1976
b (mL)	96.90b	99.00a	100.00a	100.70a	99.00a	0.63	0.0035	0.0091
c (mL/h)	0.03	0.03	0.03	0.03	0.03	0.00	0.4392	0.4151
a+b	91.00c	93.50b	95.50ab	97.50a	96.00a	0.59	<0.0001	0.0062
OMD	54.10c	56.40b	57.00ab	58.10a	58.30a	0.48	<0.0001	0.0700

a - volume of gas produced from immediate soluble fraction; b - volume of gas produced from insoluble fraction; c - gas production rate constant from insoluble fraction; a+b - potential extent of gas production; OMD - organic matter digestibility; DM - dry matter; SEM - standard error of the mean.
a-d - Different letters in each row are significantly different (P<0.05).

Table 4 - Effect of different levels of postbiotic inclusion on pH and volatile fatty acids (VFA) at 72 h of *in vitro* incubation

Parameter	Level of postbiotic inclusion (%)					SEM	Contrast, P-values	
	0 (control)	0.3	0.6	0.9	1.2		Linear	Quadratic
pH	6.76	6.76	6.64	6.76	6.77	0.05	0.9064	0.1378
Acetic (mol/100 mol)	29.20b	29.91b	31.77a	32.71a	32.79a	0.61	<0.0001	0.1531
Propionic (mol/100 mol)	9.00c	9.92b	10.17ba	10.84a	10.88a	0.46	<0.0001	0.1424
Butyric (mol/100 mol)	4.03b	4.32b	4.28b	4.94a	5.13a	0.26	<0.0001	0.4040
Total VFA (mM/L)	42.23d	44.15c	46.22b	48.49a	48.8a	0.81	<0.0001	0.1003
Acetic/propionic ratio	3.01	3.02	3.03	3.16	3.25	0.15	0.1001	0.6100

SEM - standard error of the mean.
a-d - Different letters in each row are significantly different (P<0.05).

Table 5 - Effect of different levels of postbiotic inclusion on the population of bacteria and total protozoa at 72 h *in vitro* incubation

Log_{10} microbial/mL	Level of postbiotic inclusion (%)					SEM	Contrast, P-values	
	0 (control)	0.3	0.6	0.9	1.2		Linear	Quadratic
Total bacteria	8.90c	9.22b	9.38ab	9.46a	9.49a	0.07	<0.0001	0.0172
L. plantarum	2.45	2.43	2.39	2.15	2.68	0.14	0.7642	0.2250
F. succinogenes	4.68b	4.94ab	5.09a	5.05a	5.26a	0.09	0.0010	0.4581
R. albus	6.99b	7.48a	7.52a	7.62a	7.74a	0.09	<0.0001	0.0886
R. flavefaciens	2.44b	2.73ab	3.25a	3.16a	2.84ab	0.15	0.0306	0.0072
Total protozoa	3.28c	3.48bc	3.77a	3.67ab	3.73ab	0.09	0.0006	0.0495

SEM - standard error of the mean.
a-c - Different letters in each row are significantly different (P<0.05).

Discussion

In vitro gas production and *in vivo* feed digestibility are closely associated, with gas production being the result of the fermentation of nutrients in the feed. Previous studies have reported the ability of lactic acid bacteria to survive in the rumen, change the parameters of *in vitro* rumen fermentation, and affect rumen microflora (Gollop et al., 2005; Weinberg et al., 2004). Our results indicate that the addition of postbiotics enhanced rumen fermentation rates by increasing the gas production, rumen fermentation kinetics, and OMD. The measurement of *in vitro* gas production provides useful information on the digestion kinetics of soluble and insoluble fractions of feeds (Getachew et al., 1998). The negative gas production from the soluble fraction was due to delayed fermentation because of a delay in microbial colonization or a lag period after the degradation of the soluble fraction prior to cell wall fermentation (Blümmel and Becker, 1997). Increase in net gas production, volume of gas produced from insoluble fraction, and potential extent of gas production indicate an increase in the digestibility of substrates and activity of fibre-degrading microbes. Soriano et al. (2014) reported that 1% of the supernatant from *Lactobacillus mucosae* showed no significant difference in total gas production, gas production kinetics, and OMD. The different strains of lactic acid bacteria and the varying inclusion levels may have different impacts on rumen fermentation. The role of rumen microorganisms, including bacteria and protozoa, in the digestion of soluble and insoluble feed fractions is well known. In the present study, the improvement of gas production kinetics, OMD, and VFA due to postbiotic inclusion can be explained by the improvement in the major microbial population.

In the present study, the addition of postbiotics had no impact on pH levels, but significantly increased total and individual VFA production in the rumen fluid. Acetic to propionic ratio also increased with increasing inclusion levels of postbiotics. The fibrous component of plants is the major source of carbohydrates in ruminants which is fermented by the rumen microbes to produce VFA, which are then absorbed by the rumen wall, contributing a major energy source for the host animals (Candyrine et al. 2017). Nutrient components of the *in vitro* substrate are fermented, resulting in the production of VFA and gases (mainly CO_2 and CH_4) and microbial cell growth (Getachew et al., 1998). The improvement in substrate digestion by adding postbiotics contributed to the higher production of VFA. This finding is consistent with Soriano et al. (2014), who reported a significant improvement of individual and total VFA concentrations by the inclusion of 1% of the supernatant of *L. mucosae* in *in vitro* incubation over a period of 48 h. In contrast, O'Brien et al. (2013) found that 5% (v/v) of the supernatant of *L. plantarum* TUA1490L reduced the individual and total VFA levels; the authors suggested that this was due to the presence of high concentrations of hydrogen peroxide in the supernatant. The discrepancy of these findings may be due to the differences in the bacterial strains used and the different supernatant inclusion levels. In addition, the postbiotic level of up to 1.2% in this study did not cause a significant reduction of VFA. O'Brien et al. (2013) claimed that high concentrations of hydrogen peroxide in the supernatant collected from an *L. plantarum* culture may contribute to lower VFA levels. However, hydrogen peroxide was not detected in postbiotics (Thanh et al., 2010; Thu et al., 2011a).

Our study also highlights the effect of postbiotics on the microbial population in the *in vitro* rumen fermentation. Inclusion of postbiotic increased total bacteria, cellulolytic bacteria, and total protozoa. The improvement in molar concentration of acetic acid and acetic to propionic acid ratio shows increase in the activity of cellulolytic bacteria in rumen fluid as the higher the activity of cellulolytic bacteria, the higher the molarity of acetic acid produced. *Fibrobacter*, *Ruminococcus*, and *Butyrivibrio* are the dominant cellulolytic bacteria in the rumen that degrade fibre of plant cell wall to produce VFA (Krause et al. 1999). Likewise, increase in the population of cellulolytic bacteria

could explain the higher gas production, OMD, and VFA. It is suggested that postbiotics are capable of exhibiting probiotic effects without the presence of living cells (Loh et al., 2010; Thanh et al., 2010; Thu et al., 2011b). The mechanism of lactic acid bacteria in modulating rumen microbes is still unclear. In the current study, the improvement in total bacteria and cellulolytic bacteria could be due to the interaction of postbiotics which contains metabolites of lactic acid bacteria with the rumen microbes. Weinberg et al. (2003) proposed the interaction of lactic acid bacteria with rumen microorganisms to enhance rumen fermentation and inhibition of harmful microorganisms due to the presence of antimicrobial substances such as bacteriocins by lactic acid bacteria. Yoon and Stern (1995) reported that the stimulation of microbial growth, changes in the fermentation pattern in the rumen, and improvement of the digestibility are the modes of action of probiotics supplemented in ruminant diets. Probiotic supplementation has been suggested to enhance the adaptation of ruminal microbes to the presence of lactic acid or to impede the lactic acid accumulation in the rumen by lactic acid degradation to acetic acid (Ghorbani et al., 2002; Nocek et al., 2002). Jiao et al. (2017) proposed that these conditions favour the activities of cellulolytic bacteria and increase microbial digestion of fibrous feeds. This is consistent with the current study, in which the increment of total bacteria and fibre-degrading bacteria improved OMD levels when postbiotics were included.

Information on the effects of postbiotics on the rumen microbial population, including ruminal protozoa, is scarce. The role of protozoa in the rumen is still unclear, but one study reports that elimination of the protozoa (defaunation) increases the microbial protein supply and reduces methane production (Newbold et al., 1986). The increase in the number of protozoa in the current study could be due to the presence of lactate in the postbiotics; protozoa consume lactate more rapidly than bacteria, resulting in higher lactate levels in defaunated animals (Newbold et al., 1986; Williams and Coleman, 1997).

Conclusions

The inclusion of postbiotics positively affects *in vitro* rumen fermentation parameters. The populations of cellulolytic bacteria, such as *F. succinogenes*, *R. albus*, and *R. flavefaciens* and protozoa, increase with the treatment with postbiotics. This indicates a higher fibre digestion, which may contribute to increased rumen fermentation.

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