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Effects of Nisin and Propolis on Ruminal Fermentation *in vitro*

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Abstract: The objective of this study was to investigate the effects of nisin and propolis on *in vitro* fermentation of a 60:40 forage:concentrate diet using the rumen simulation technique and to compare their effects with the antibiotic monensin (positive control). Nisin, propolis ethanol extract and monensin were added daily at 2 mg, 100 μL (contained active substances of 2 mg crude propolis) and 5 mg to the fermentation vessels, respectively. Monensin caused expected changes in fermentation patterns (a significant decrease in NH_3-N concentration, acetate and butyrate production, protozoa counts and a significant increase in propionate production). Nisin and propolis did not cause beneficial effects on fermentation efficiency except NH_3-N concentration. NH_3-N concentration was reduced ($p<0.05$) by them. In conclusion, the results of this study indicate that nisin and propolis might be useful additives to decrease ruminal ammonia production and to improve nitrogen utilization by ruminants.

Key words: Nisin, propolis, nitrogen utilization, rumen, protozoa, monensin

INTRODUCTION

Ruminant animals have a symbiotic relationship with their ruminal microflora (bacteria, protozoa and fungi). The ruminant provides the microorganisms with a habitat for their growth and microorganisms supply the animal with fermentation acids, microbial protein and vitamins. However, ruminal fermentation has energy (losses of methane) and protein (losses of ammonia N) inefficiencies (Van Nevel and Demeyer, 1988). Therefore, specific manipulation of the ruminal microbial population to increase the fermentation efficiency has long been a goal of ruminant nutritionists and microbiologists. Antibiotic growth promoters have been successfully used to improve ruminal fermentation. However, due to the risk of transferring residues into meat and milk and resistant strains of bacteria, the use of antibiotics in animal nutrition has been prohibited in the European Union since January 2006 (Oeztuerk and Sagmanligil, 2009). For this reason, attention has recently shifted to evaluate other safe alternatives.

Nisin is a natural, toxicologically safe, antibacterial food preservative. It is regarded as natural because it is a polypeptide produced by certain strains of the food-grade lactic acid bacterium *Lactococcus lactis* subsp. *lactis*, during fermentation. Nisin exhibits antimicrobial activity towards a wide range of Gram positive bacteria and is particularly effective against spores (Delves-Broughton, 2005). Propolis is a resinous substance collected by honeybees from buds and leaves of trees and plants, mixing with pollen as well as enzymes secreted by bees.

Substances which are identified in propolis generally are typical constituents of food and/or food additives and are recognized as GRAS (Generally Recognized As Safe) substances (Burdock, 1998). The antimicrobial action of propolis has been reported previously and the results of these studies have shown that Gram positive bacteria are more susceptible to antibacterial action of propolis than Gram negative bacteria (Gonsales *et al*., 2006). Gram positive bacteria produce more ammonia, hydrogen and lactate than Gram negative species and compounds that inhibit Gram positive ruminal bacteria have increased feed efficiency (Russell and Strobel, 1989).

The effects of nisin and propolis on rumen microbial fermentation have not been widely assessed. The objective of the present study was to evaluate the potential benefits of nisin and propolis as modifiers of rumen microbial fermentation in a long-term *in vitro* study.

The ionophore antibiotic monensin was also used as a positive control to compare its effects with those of nisin and propolis in the same *in vitro* conditions.

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MATERIALS AND METHODS

Incubation technique: The Rumen Simulation Technique (RUSITEC) as described by Czerkawski and Breckenridge (1977) was used. The experiment consisted of nine plexiglass 1000 mL vessels maintained at constant temperature (39°C). These fermentation vessels were inoculated on day 1 with liquid (gauze-filtered) and solid rumen contents taken from two freshly slaughtered adult sheep (55 kg mean body weight) and transferred to the *in vitro* system within 30 min.

Animals had been fed a diet of alfalfa hay *ad libitum* and 1 kg of pelleted concentrate per day. The same diet was also used for *in vitro* incubation trial. Each vessel was loaded with 2 nylon bags (70×120 mm with a pore size of 150 μm). At the start of the study, one bag was filled with 80 g of solid rumen contents (fresh weight) and the other with the daily diet, a mixture of 4 g of pelleted concentrate and 5 g of alfalfa hay cut into 0.5 cm lengths. The compositions of the alfalfa hay and pelleted concentrate were shown in Table 1.

The nylon bag with solid rumen contents was replaced after 24 h of incubation with a bag containing the diet. The feed bag was changed after 48 h so that 2 bags were always present. This gave a retention time of 48 h for feed. When the bag was being changed, the vessels were flushed with nitrogen to maintain anaerobic conditions. The liquid flow through the vessels was maintained by continuous infusion of a buffer solution at a rate of 750 mL day^{-1}. The pH was 7.4 and the osmolality was 293 mosmol L^{-1}. The chemical composition of the buffer solution was shown in Table 2.

Experimental procedure: The incubation trial consisted of three periods, an equilibration period, period 1 and period 2 (7 days for each). The first 7 days were allowed for equilibration of the system to achieve steady state conditions. The following 7 days (period 1) were used to determine fermentation parameters under control conditions without any supplementation (negative control). The last 7 days (period 2) represented an experimental period during which monensin, nisin and propolis were added to the respective fermentation vessels. In this last period, nine fermentation vessels were divided into 3 groups with three vessels in each group. The first group received daily 5 mg of monensin (monensin sodium, Fluka) and served as positive control. The second and third groups received daily 2 mg of nisin (2.5%, Sigma Chemical Co.) and 100 μL of propolis extract (contained active substances of 2 mg crude propolis), respectively. Because propolis is almost insoluble in water and soluble in organic solvents such as ethyl alcohol, an ethanolic extract of propolis was used in this study. Crude propolis was ground into a fine powder and thereafter 2% ethanolic extract of propolis was prepared (200 mg propolis powder was completed to 10 mL with 70% ethyl alcohol), protected from light with moderate shaking at room temperature. After a week, the insoluble fraction was separated by filtration. The filtrate was named ethanolic extract of propolis and was maintained in caramel flask in dark at room temperature. The *in vitro* doses of monensin (5 mg L^{-1}) and nisin (2 mg L^{-1}) used in this study were based on previously published reports (Callaway *et al*., 1997; Jalc and Laukova, 2002). The dose of propolis (2 mg L^{-1}) was chosen as similar to the *in vitro* doses of monensin and nisin due to deficiency of literature about the usage of propolis in ruminants.

Table 1: Chemical composition of the experimental diet (g kg^{-1} of DM)

Ingredient	Alfalfa hay	Concentrate
Dry matter	915	875
Crude protein	148	176
Crude fibre	269	54
NDF	470	210
ADF	300	121
Ash	95	72

Table 2: Chemical composition of the buffer solution

Ingredient	(mmol L^{-1})
NaCl	28.00
KCl	7.69
$CaCl_2.2H_2O$	0.22
$MgCl_2.6H_2O$	0.63
NH_4Cl	5.00
$Na_2HPO_4.12H_2O$	10.00
$NaH_2PO_4.H_2O$	10.00
$NaHCO_3$	97.90

Analytical procedures and samplings: The pH values were measured daily in each vessel at the time of feeding using an epoxy body pH electrode (WD-35801-00, Oakton) connected to a pH-meter (Ion 6, Acorn series, Oakton). About 5 mL of liquid effluents collected in ice-cold flasks were taken daily and immediately acidified with 0.4 mL of dilute HCl (9.25%) and frozen at -20°C until analysed for ammonia nitrogen. Samples of the effluents were also collected daily and kept at -20°C until SCFA (short-chain fatty acids) analysis. Ruminal SCFA samples were allowed to thaw completely at 4°C before analysis. Samples were then acidified (pH<3) with 90 μL of 12 N H_2SO_4, vortexed and centrifuged (Universal 32R, Hettich Zentrifugen, Germany) in Eppendorf tubes for 30 min at 13000 rpm. The supernatant was filtered through a 0.2 μm PTFE membrane (Millex-GN, Millipore). Concentrations of SCFA in the supernatant were then determined by HPLC (Dionex Summit P680, ASI100) equipped with an UV absorbance detector (Dionex UVD170) operated at 210 nm. Separation of acids was conducted using an organic acid analysis column (300×7.8 mm; Rezex ROA-Organic Acid

column) with 0.005 M H_2SO_4 as eluent at flow rate of 0.6 mL min^{-1} and with the column temperature of 60°C. A Rezex ROA Organic Acid precolumn (50×7.8 mm) was used to protect the column from any particles that could have been injected together with the samples. Daily production rates of SCFA were estimated by multiplying the respective concentration by the volume of effluent collected.

Ruminal NH_3-N samples were allowed to thaw completely at 4°C before analysis. NH_3-N concentrations were determined by means of an ammonia gas sensing electrode (Ammonia combination electrode, Cole-Parmer; calibrated daily with serial dilutions of an NH_4Cl stock solution) connected to an Acorn series Ion meter (Oakton Instruments, USA).

For bacteria and protozoa counting, rumen fluid samples of fermentation vessels were taken daily immediately before substrate exchange. For protozoa counting, 1 mL of sample was carefully mixed with 1 mL of a solution of 0.6 g methyl green, 6 g NaCl and 100 mL formaldehyde (37%) filled up to 1000 mL aqua dest. Portions of the samples were then pipetted into a counting chamber (Fuchs-Rosenthal: 0.0625 mm^2; 0.2 mm deep; Marienfeld, Germany). Total numbers of protozoa without quantifying different types were determined using a light microscope (Leica CME). For bacteria counting, 0.1 mL rumen fluid was mixed with 0.9 mL 37% formaldehyde. Direct counts of total bacteria were made using a cell chamber (Thoma: 0.0025 mm^2 squares, 0.02 mm deep; Brand, Germany) under phase-contrast microscope (Olympus Optical Co., Japan).

Dry matter was determined by drying at 65°C for 48 h. The digestibility of dry matter at 48 h was calculated as original dry matter sample weight minus dry matter residue weight divided by the original sample weight. This value was then multiplied by 100 to derive the digestibility of dry matter percentage.

Statistical analyses: Results are given as least squares means and pooled Standard Error of the Mean (SEM); n designates the number of fermentation vessels run in parallel. Statistical analysis was performed by a two-way Analysis of Variance (ANOVA) using the SigmaStat 3.1 software (Systat Software, Erkrath, Germany) with treatment and time as well as their interaction as factors of variance. In case of a significant ANOVA result, post hoc Duncan tests were performed to evaluate the statistical differences between the groups. Constancy of equilibrium conditions during seven-day control period was tested by one-way ANOVA. None of the parameters were significantly affected during the control period. Therefore, mean values of the 7 day control measurements in the respective number of fermentation vessels served as control values to test treatment effects of monensin, nisin and propolis. $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Throughout the experiment, pH values ranged between 6.74 and 6.83 within the physiological range of rumen fluid pH. Mean pH values were significantly ($p<0.05$) increased from a control value of 6.77-6.80 and 6.79 in the presence of nisin and propolis, respectively (Fig. 1). However, the addition of monensin did not significantly influence ruminal pH (Fig. 1).

The mean production of total SCFA were significantly ($p<0.05$) decreased from 34.25 mmol day^{-1} during the control period to 31.86 mmol day^{-1} in response to nisin (Fig. 2). In general, these reductions were mediated by respective changes in the production rates of acetate and propionate (Fig. 2 and 3). However, no reductions in the production rates of total SCFA were recorded in the presence of monensin and propolis

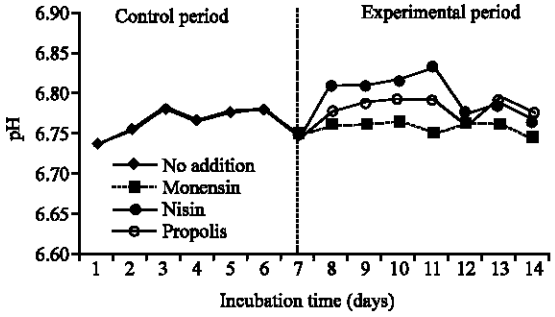

Fig. 1: Effects of monensin, nisin and propolis on ruminal pH in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment); 2-way ANOVA shows effect of treatment: $p<0.05$; Time: NS; Interaction: NS; SEM: 0.01

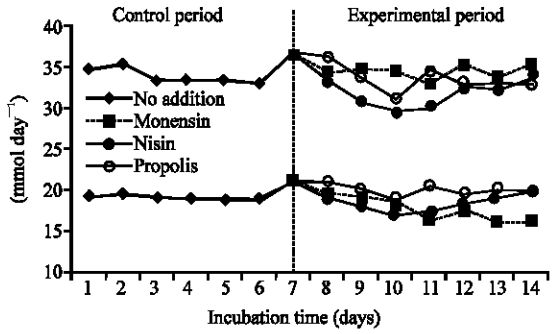

Fig. 2: Effects of monensin, nisin and propolis on total-SCFA and acetate production in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment); 2-way ANOVA shows effect of total-SCFA and acetate in treatment ($p<0.05$, 0.05); Time: (NS, NS); Interaction (NS, NS); SEM (0.49, 0.30), respectively

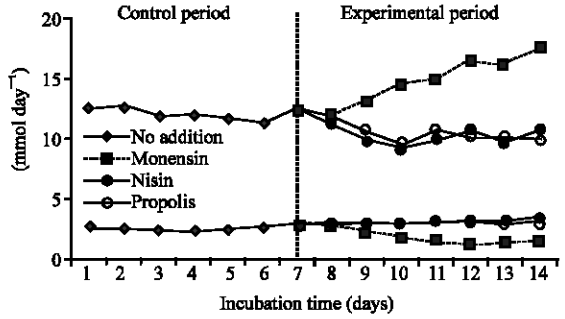

Fig. 3: Effects of monensin, nisin and propolis on propionate and butyrate production in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment); 2-way ANOVA shows effects of propionate and butyrate in treatment ($p<0.05$, 0.05) Time (NS, NS); Interaction (NS, NS); SEM (0.34, 0.09), respectively

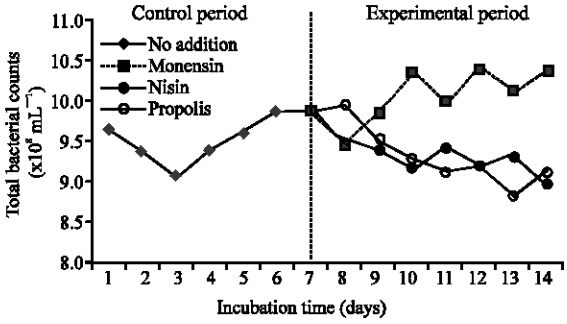

Fig. 4: Effects of monensin, nisin and propolis on total bacterial counts in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment); 2-way ANOVA shows effects of treatment: $p<0.05$; Time: NS; Interaction: $p<0.05$; SEM: 0.09

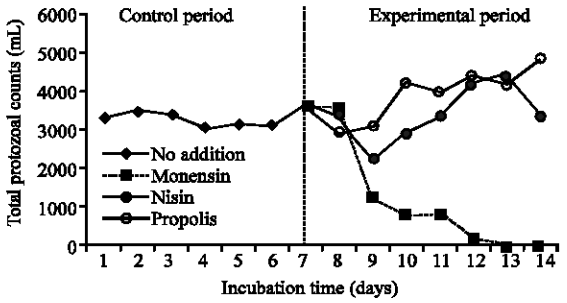

Fig. 5: Effects of monensin, nisin and propolis on total protozal counts in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment) 2-way ANOVA shows effects of treatment: $p<0.05$; Time: NS; Interaction: $p<0.05$; SEM: 242.14

(Fig. 2). Production rates of the individual SCFA were distinctly affected. Acetate production decreased ($p<0.05$) from a control level of 19.44 mmol day^{-1} by 5%-18.47 mmol day^{-1} after nisin addition. Compared with the unsupplemented control period, the reduction in acetate production was approximately 10% in the presence of monensin (19.44 versus 17.60 mmol day^{-1}; $p<0.05$). However, propolis did not affect acetate production (Fig. 2).

Significant decreases ($p<0.05$) in propionate production from 12.13 mmol day^{-1} during the 7 days control period to 10.24 and 10.45 mmol day^{-1} (-14%) were recorded in response to nisin and propolis, respectively (Fig. 3). In contrast, production rate of propionate was significantly ($p<0.05$) increased by 24% in the presence of monensin (12.13 versus 15.05 mmol day^{-1}; Fig. 3). Thus, the ratio of acetate to propionate increased significantly ($p<0.05$) from 1.61-1.82 and 1.93 by nisin and propolis, respectively.

This ratio was significantly ($p<0.05$) decreased from 1.61-1.20 by monensin treatment. The addition of monensin resulted in a significant decrease ($p<0.05$) in butyrate production from 2.69 mmol day^{-1} by 32%-1.82 mmol day^{-1}. However, in the presence of nisin and propolis, production rates of butyrate were significantly ($p<0.05$) increased from the control value of 2.69-3.15 and 3.00 mmol day^{-1}, respectively (Fig. 3).

Total bacterial counts did not show significant alterations in the presence of nisin and propolis (Fig. 4). Mean total counts were 9.54×10^8 mL^{-1} rumen fluid during the unsupplemented control period and 9.29×10^8 mL^{-1} rumen fluid during the experimental period in which nisin and propolis were added to the respective fermentation vessels. Addition of monensin caused a small but significant ($p<0.05$) increase in total bacterial counts (9.54×10^8 versus 10.01×10^8, Fig. 4). On the other hand, monensin resulted in a significant decrease ($p<0.05$) in total protozoal counts. Furthermore, no protozoa were detected after 5 days of monensin treatment. Additions of nisin and propolis did not affect total protozoal counts (Fig. 5). Total numbers of rumen protozoa ranged between 3303 mL^{-1} rumen fluid during the control period and 3392 and 3928 mL^{-1} rumen fluid in the presence of nisin and propolis, respectively.

NH_3-N concentration decreased (treatment $p<0.05$, time $p<0.05$, interaction $p<0.05$) from a control value of 11.22-6.95, (-38%), 9.33, (-17%) and 9.48 mmol L^{-1} (-16%) in the presence of monensin, nisin and propolis, respectively (Fig. 6). Digestibility of dry matter was not significantly affected by all three antimicrobial substances tested here.

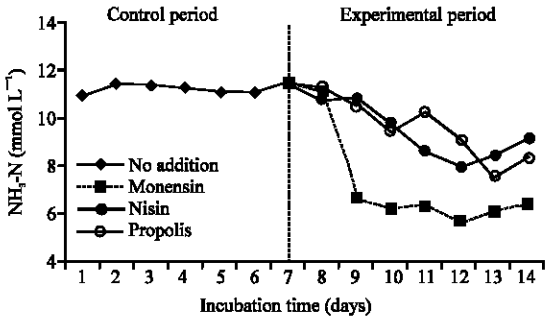

Fig. 6: Effects of monensin, nisin and propolis on NH_3-N concentration in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment). 2-way ANOVA shows effects of treatment: $p<0.05$; Time: $p<0.05$; Interaction: $p<0.05$; SEM: 0.20

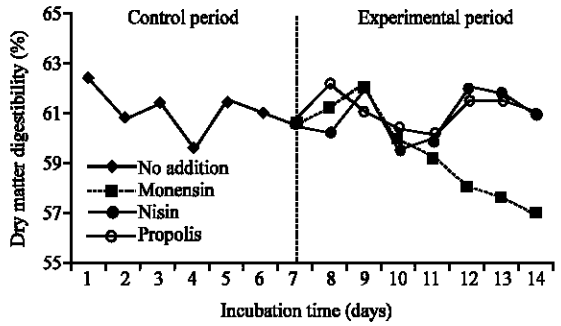

Fig. 7: Effects of monensin, nisin and propolis on dry matter digestibility in Rusitec, n = 9 (control period), n = 3 (for each antimicrobial treatment). 2-way ANOVA shows effects of Treatment: NS; Time: NS; Interaction: $p<0.05$; SEM: 0.77

However, because of a trend toward a decrease in dry matter digestibility by monensin treatment, a significant effect for interaction between treatment and time was recorded (Fig. 7).

Throughout the experiment, pH values ranged between 6.74 and 6.83 and were thus within the physiological range of rumen fluid pH. Compared with the control period, the significant and numerical increases in ruminal pH after adding nisin and propolis but not monensin may reflect low SCFA production in these treatments. In the present study, monensin reduced the production of acetate and butyrate and the ratio of acetate to propionate and increased the production of propionate without affecting total SCFA production. These results are in agreement with other *in vitro* (Wallace *et al*., 1981) and *in vivo* (Sauer *et al*., 1998) studies. In the current study, expected changes in the production and the profile of SCFA (decreases in acetate and butrate production and acetate:propionate ratio and an increase in propionate production) was not recorded by the treatment of nisin and propolis. Effects of nisin and propolis on the production and the profile of ruminal SCFA are limited and contradictory in the literatures so far. In an *in vitro* experiment conducted by Callaway *et al*. (1997), nisin doses of >5.7 mg L^{-1} decreased the production of total SCFA during fermentation of alfalfa hay after 24 h of incubation which is in agreement with the results. However, Sar *et al*. (2005) reported that additions of nisin (up to 86 mg L^{-1}) increased the production rate of total SCFA in *in vitro* cultures of mixed rumen microorganisms using a diet containing oaten hay, alfalfa hay cube and concentrates (35:35:30). In another *in vitro* study conducted by Jalc and Laukova (2002), the addition of 2 mg day^{-1} of nisin increased acetate and propionate production without affecting total SCFA production in the Rusitec fermenters received a 80:20 meadow hay:crushed barley diet. Broudiscou *et al*. (2000) found that an addition of 0.5 g L^{-1} of propolis extract increased propionate production without affecting other ruminal fatty acids in dual outflow fermenters supplied with a 50:50 orchard grass hay plus barley diet. The reasons for these inconsistencies in the results concerning the SCFA production are not clear. The inconsistencies may partly be explained by three factors; confounding effects of ration composition, different doses used and different sensitivities of rumen microorganisms to nisin and propolis comparing with monensin. Jalc and Laukova (2002) underlined that the mode of action of nisin on SCFA production was different from the effect of monensin.

In the present study, monensin reduced the total numbers of protozoa in rumen fluid which is consistent with its antiprotozoal effect (Hino and Russell, 1986). However, the addition of nisin and propolis to the Rusitec vessels did not affect the total counts of rumen protozoa when compared with the unsupplemented control period. Kisidayova *et al*. (2003) showed that *Entodinium caudatum* was relatively resistant to nisin concentration up to 400 mg L^{-1} during short-term *in vitro* treatment (5 days) which is consistent with the result. Broudiscou *et al*. (2000) showed that propolis (0.5 g L^{-1}) did not significantly change the counts of rumen ciliates in dual outflow fermenters. The reason of no beneficial changes in the SCFA profile in the present study could probably be due to the fact that nisin and propolis had no effects on rumen ciliates. The complete removal of protozoa from the rumen (defaunation) shifts the fermentation pattern to increased propionate production associated with lower acetate production (Kreuzer *et al*., 1986). In the current study, the total number of ruminal

bacteria was not changed by the addition of nisin and propolis. In an *in vitro* study, Kisidayova *et al.* (2003) determined the effect of long-term nisin treatment (100 mg L^{-1}) on certain bacterial population in the protozoan cultures. They found that Gram positive facultative anaerobic bacterial population was inhibited in the presence of nisin. No reports were found in the literature about the effects of propolis on ruminal bacteria. In this study, the reason of no inhibition of ruminal bacteria count might be due to low doses of both nisin and propolis. Interestingly, monensin caused a small but statistically significant increase in the total bacterial counts. This effect could be associated with the antiprotozoal effect of monensin. Ciliate protozoa engulf and digest rumen bacteria, thereby regulating bacterial number in the rumen. Predation by ciliate protozoa can account for 90% of the bacterial protein turnover in the rumen (Wallace and McPherson, 1987). Similarly to the results, Wallace *et al.* (1981) found that monensin (2, 10, 50 mg day^{-1}) increased numerically the total bacterial counts from 3.9×10^{10}-6.3×10^{10} when it was added to the hay-barley (70:30%) diet in Rusitec.

The present experiment showed that NH_3-N concentrations decreased when ruminal fluid was incubated with monensin, nisin and propolis which is consistent with previous reports (Callaway *et al.*, 1997; Oliveira *et al.*, 2006). However, the decrease by nisin and propolis was less than that by monensin. This suggests that amino acid fermenting bacteria could be more sensitive to monensin than that to nisin and propolis. In a study with Holstein cows, Yang and Russell (1993) demonstrated that up to 50% decrease in rumen ammonia caused by monensin was associated with a 10 fold decrease in the number of obligate amino acid fermenting bacteria.

Nisin and propolis did not significantly affect dry matter digestibility of diets incubated for 48 h in the Rusitec apparatus. Towards to the end of the experiment however, dry matter digestibility was numerically reduced by the treatment of monensin. The negative effects of monensin on diet digestion agree with other *in vitro* studies (Wallace *et al.*, 1981; Russell and Strobel, 1988). This effect of monensin on diet digestion was attributed to the higher sensitivity of cellulolytic ruminococci and other ruminal cellulolytic strains to ionophores (Russell and Strobel, 1989).

CONCLUSION

The results obtained from this study showed that nisin and propolis were less effective than monensin as modifiers of ruminal fermentation. In particular, these antibacterial substances had no beneficial effects on the production and the profile of ruminal SCFA as well as on the ciliate protozoa. Considering these results, it can be suggested that the doses used for nisin and propolis might have been too low and/or the sensitivities of rumen microorganisms to these antimicrobials could be different from that of monensin. On the other hand, the inhibition effect of nisin and propolis on ruminal ammonia production may be helpful to improve the nitrogen retention in ruminants. Further *in vitro* and *in vivo* researches with different doses and diet formulations are needed to evaluate the use of nisin and propolis as modifiers of rumen microbial fermentation.

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