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Comparative effects of nisin and monensin on pure cultures of rumen bacteria

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ABSTRACT:

Nisin is a low molecular weight bacteriocin which is produced by *Lactococcus lactis*. Literature about the effects of nisin on pure cultures of rumen bacteria is scarce. The objective of the present study was to investigate the comparative effects of nisin and monensin on pure cultures of some Gram-positive and Gram-negative rumen bacteria. The antibacterial activity assays of nisin and monensin were carried out using broth microdilution method in anaerobic conditions. Antibacterial effect of monensin on Gram-positive rumen bacteria was higher than nisin. *Ruminococcus albus* and *Eubacterium ruminantium* were the most sensitive bacteria to monensin. Growth of these bacteria was inhibited completely by monensin, at 6 and 12 µg/mL concentrations respectively. Nisin exhibited stimulatory effects on *R. albus*, *E. ruminantium* and, *Streptecoccus bovis* ($p<0.05$), unlike monensin. Both nisin and monensin showed potential antibacterial activity on *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens* ($p<0.05$), although they did not inhibit these bacteria completely. Nisin and monensin also did not show inhibitory effect on *Methanobacterium formicicum*, however the potential antimicrobial activity of monensin on this methanogen was more evident. Gram-negative rumen bacteria, *Megasphaera elsdenii* and *Fibrobacter succinogenes*, were resistant to both of nisin and monesin. It was concluded that the different effects of nisin and monensin particularly on some Gram-positive rumen bacteria may be responsible for their inconsistent effects on ruminal fermentation.

Nisin ve monensin'in rumen bakterilerinin saf kültürleri üzerine karşılaştırmalı etkileri

ÖZET:

Nisin, *Lactococcus lactis* tarafından üretilen düşük moleküler ağırlıklı bir bakteriyosindir. Nisin'in rumen bakterilerinin saf kültürleri üzerine etkileri hakkında oldukça sınırlı düzeyde literatür bilgisi bulunmaktadır. Bu çalışmanın amacı, nisin ve monensin'in bazı Gram-pozitif ve Gram-negatif rumen bakterilerinin saf kültürleri üzerine karşılaştırmalı etkilerini araştırmaktır. Nisin ve monensin'in antibakteriyal aktivite analizleri, anaerobik koşullarda mikrodilüsyon yöntemi kullanılarak yapılmıştır. Monensin'in Gram-pozitif rumen bakterileri üzerine antibakteriyal etkisi nisin'den daha yüksek bulunmuştur. *Ruminococcus albus* ve *Eubacterium ruminantium* monensin'e karşı en duyarlı bakteriler olarak belirlenmiştir. Bu bakterilerin büyümesi, monensin tarafından sırasıyla 6 ve 12 µg/mL konsantrasyonlarında tamamen baskılanmıştır. Nisin, monensin'den farklı olarak *R. albus*, *E. ruminantium* ve *Streptecoccus bovis* üzerine uyarıcı etkiler göstermiştir ($p<0,05$). Hem nisin hem de monensin, *Ruminococcus flavefaciens* ve *Butyrivibrio fibrisolvens* üzerine potansiyel antibakteriyal aktivite sergilemişler ($p<0,05$), ancak bu bakterileri tamamen baskılamamışlardır. Nisin ve monensin ayrıca *Methanobacterium formicicum* üzerine baskılayıcı etki göstermemekle birlikte, monensin'in bu metanojen üzerine potansiyel antimikrobiyal etkinliğinin daha belirgin olduğu gözlenmiştir. Gram-negatif rumen bakterileri olan *Megasphaera elsdenii* ve *Fibrobacter succinogenes*'in, hem nisin hem de monensin'e karşı dirençli oldukları belirlenmiştir. Nisin ve monensin'in özellikle bazı Gram-pozitif rumen bakterileri üzerine olan farklı etkilerinin ruminal fermentasyon üzerine uyumlu olmayan etkilerinden sorumlu olabileceği sonucuna varılmıştır.

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1. Introduction

Ruminants, thanks to their rumen and its microorganisms, are able to process indigestible feedstuffs and convert them in nutritious food. Rumen bacteria, which make up 95% of the total microbiota in the rumen (3), break down plant materials and form volatile fatty acids and bacterial proteins as evaluable energy and protein sources for the host animal (14). However, Gram-positive species which produce greater amounts of hydrogen, formate, ammonia, and lactic acid compared to the Gram-negative bacteria in the rumen lead to the loss of energy and protein (5). Ionophore antibiotics that are affect selectively Gram-positive bacteria improve animal productivity via increasing propionate production, fibre digestion and decreasing methanogenesis, deamination of amino acids, proteolysis of dietary crude protein, and accumulation of lactate (26). Monensin is the most commonly used ionophore until the antibiotic ban for feed additives in 2006 (21).

Many lactic acid bacteria produce antibacterial small peptides, called as 'bacteriocins', which are primarily effective against Gram-positive bacteria (12). Nisin is a low molecular weight bacteriocin (lantibiotic) which is produced by *Lactococcus lactis*, a common starter culture for cheese making (10). Nisin also has "generally recognized as safe" status, and is approved for use as a food preservative (12). It is known that nisin shows antimicrobial activity against a wide range of Gram-positive bacteria such as *Listeria monocytogenes*, *Bacillus cereus* (24), *Streptococcus pneumoniae* and, *Staphylococcus aureus* (28). It is suggested that nisin might have potential to modify ruminal fermentation because of its ability to inhibit Gram-positive ruminal bacteria (4). Callaway et al. (4) reported that even low concentrations of purified nisin inhibited *in vitro* methane production, decreased the acetate to propionate ratios and reduced ammonia production similar to monensin. On the other hand, there are also reports indicate that nisin was less effective than monensin (13, 20) as well as, monensin and nisin affect rumen fermentation and microbiota differently *in vitro* (16, 29). However, literature about the effects of nisin on pure cultures of rumen bacteria is scarce. Such an information can contribute to the clear physiological mechanisms and the mode of action of nisin in the rumen. Therefore, the objective of the present study was to investigate the comparative effects of nisin and monensin on pure cultures of some Gram-positive and Gram-negative rumen bacteria.

2. Material and Methods

Nisin and monensin:

Nisin was obtained from Sigma Aldrich (N5764), and monensin was obtained from Fluka (69864).

Bacterial strains:

The Gram-positive bacterial species used in antimicrobial tests were *Ruminococcus albus* (ATCC 27210) and *Ruminococcus flavefaciens* Sijpestejin C97 (ATCC 49949) as hydrogen and formate producers, *Butyrivibrio fibrisolvens* D1 (ATCC 19171) and *Eubacterium ruminantium* GA 195 (ATCC 17233) as butyrate producers, and *Streptococcus bovis* (ATCC 33317) as a lactate producer. *Methanobacterium formicicum* (ATCC 33274), a mesophilic methanogen, was used as a methane producer. The Gram-negative bacterial species tested were *Fibrobacter succinogenes* S85 (ATCC 19169) and *Megasphaera elsdenii* LC1 (ATCC 25940), which were used as succinate and propionate producers.

Anaerobic media:

Growth media for bacterial cultures were prepared under CO_2 to maintain anaerobic conditions according to Orpin (22). The chemical composition of anaerobic media is shown in Table 1. The media was gassed with CO_2 while heating to 60 °C in a hot water bath to remove O_2 completely. The conversion of the color of medium to dull yellow from bluish purple by the resazurin (0.1%, v/v), which is a redox potential indicator in the medium, was considered to be a sign of removal of oxygen. Bottle of media was closed with a rubber stopper and autoclaved.

Anaerobic bacteria were grown at 37 ºC for 24-72 h under strictly anaerobic conditions inside an anaerobic chamber (Whitley DG250, Don Whitley, West Yorkshire, UK) under an atmosphere of N_2-CO_2-H_2 (80:10:10).

Table 1: Composition of the anaerobic media (for 100 mL) (Orpin 1976)

Tablo 1: *Anaerobik besiyerinin bileşimi (100 mL için) (Orpin 1976)*

Component*	
Mineral solution 1**	15
Mineral solution 2***	15
Clarified rumen fluid****	15
$NaHCO_3$ (Sigma S5761)	0.6
Yeast extract (Sigma Y1625)	0.25
Trypticase peptone (BD 211921 Bacto™)	1
Resazurin (%0.1, v/v) (Sigma R7017)	1
Cysteine HCl (Sigma C7880)	0.1
Cellobiose (Sigma 22150)	0.5
Deionized water	55

**Units are mL for liquid components and g for solid components.*

***Mineral solution 1: 3 g/L K_2HPO_4 (Sigma P3786)*

****Mineral solution 2: 3 g/L KH_2PO_4 (Sigma P9791), 6 g/L $(NH_4)_2SO_4$ (Sigma A4915), 6 g/L NaCl (Sigma S7653), 0.6 g/L $MgSO_4 \bullet 7H_2O$ (Sigma 230391) and 0.6 g/L $CaCl_2$ (Sigma C1016)*

*****The ruminal fluid brought from the slaughterhouse was mixed and filtered through three layers of cheesecloth to partition into liquid and solid (digesta) fractions. Liquid fraction was centrifuged at 15000 rpm and, the clear supernatant was used as a component of anaerobic media.*

Evaluation of antibacterial activity:

The antibacterial activity assays of nisin and monensin were carried out using a broth microdilution method following the Clinical and Laboratory Standards Institute guidelines (8) in the anaerobic chamber. Stock solutions of nisin and monesin (20 mg/mL) was prepared dissolving nisin and monesin in 50 % (v/v) ethanol. The minimal inhibitory concentrations (MICs) of the several ionophore and non-ionophore antibiotics on rumen bacteria were investigated at doses ranging from 0.09 to 48 µg/mL (19). Therefore, the highest concentration of samples in the present study was 48 µg/mL. A serial 2-fold dilution of nisin and monesin (48, 24, 12, 6, 3, 1.5, 0.75, 0.38, 0.19, 0.09 µg/mL) was prepared in the anaerobic media. Two hundred microliters of each was added to wells of a 96-well plate (Corning 3599, Flat bottom). Then, 20 µL aliquots of 4×10^{10} cell/mL bacteria were added into each well. Triplicate wells were used for each concentration. Negative control wells without antimicrobial compounds and media control wells without bacteria were maintained for each set. After incubation at 37 °C for 24 h in the anaerobic chamber, microbial growth was determined at 600 nm using a plate reader (BioTek, Epoch). The MIC was the lowest concentration at an OD_{600} value of ≤ 0.1 (15). A significantly lower OD_{600} value compared to control dose (0 µg/mL) was accepted as potential antibacterial activity (17) while significantly higher OD_{600} value was accepted as stimulatory activity.

Statistical analyses:

Statistical analysis was carried out by the use of one-way ANOVA followed by Dunnett's test. Each well of a 96-well plate was an experimental unit. A value of $p<0.05$ was taken to indicate a significant difference.

3. Results

Effects of nisin and monensin on rumen bacteria are presented in Figure 1 and Figure 2. Antibacterial effect of monensin on Gram-positive rumen bacteria was higher than nisin. Nisin showed growth stimulatory effect on *R. albus* at 0.09-24 µg/mL doses ($p<0.05$), while potential antibacterial activity was observed at 48 µg/mL ($p<0.05$). On the other hand, *R. albus* was inhibited completely by monensin at 6 µg/mL (MIC). Monensin also inhibited the growth of *E. ruminantium* at 12 µg/mL dosage (MIC), unlike nisin, which had stimulatory effect on this bacterium at 1.5-48 µg/mL doses ($p<0.05$). Similarly, monensin showed potential antibacterial activity against *S. bovis* at 3-48 µg/mL ($p<0.05$), while nisin promoted the growth of this bacterium at all doses ($p<0.05$). Both nisin and monensin exhibited potential antibacterial activity on *R. flavefaciens* and *B. fibrisolvens* ($p<0.05$), although they did not inhibit these bacteria completely. Nisin and monensin also did not show growth inhibitory effect on *M. formicicum*, however the potential antimicrobial activity of monensin was more evident on this methanogen.

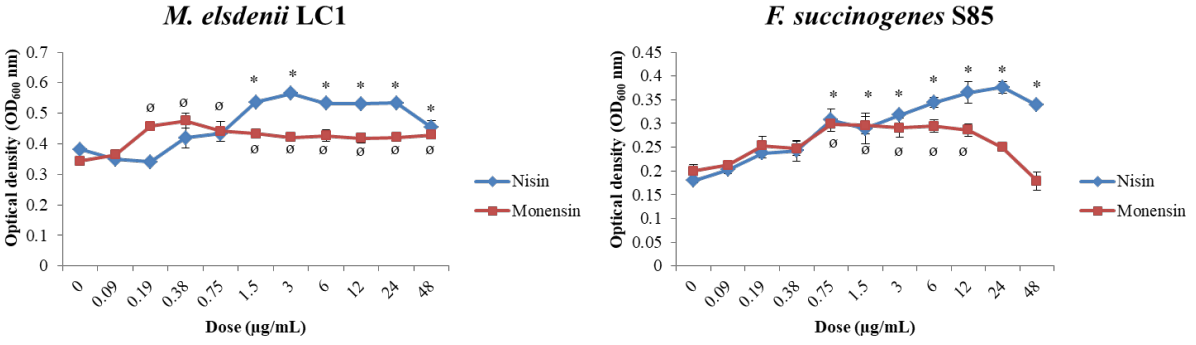

Figure 1: Effects of nisin and monensin on Gram-negative rumen bacteria. The results represent the mean ± standard error. *$p<0.05$, difference of the nisin-treated culture compared with the control; º$p<0.05$, difference of the monensin-treated culture compared with the control. Control level was 0 µg/mL for both nisin and monensin.

Şekil 1: *Nisin ve monensin'in Gram-negatif rumen bakterileri üzerine etkileri. Sonuçlar, ortalama ± standart hatayı temsil etmektedir. *$p<0,05$, nisin ile muamele edilmiş kültürün kontrol ile karşılaştırıldığında farkı; º$p<0,05$, monensin ile muamele edilmiş kültürün kontrol ile karşılaştırıldığında farkı. Kontrol seviyesi hem nisin hem de monensin için 0 µg/mL'dir.*

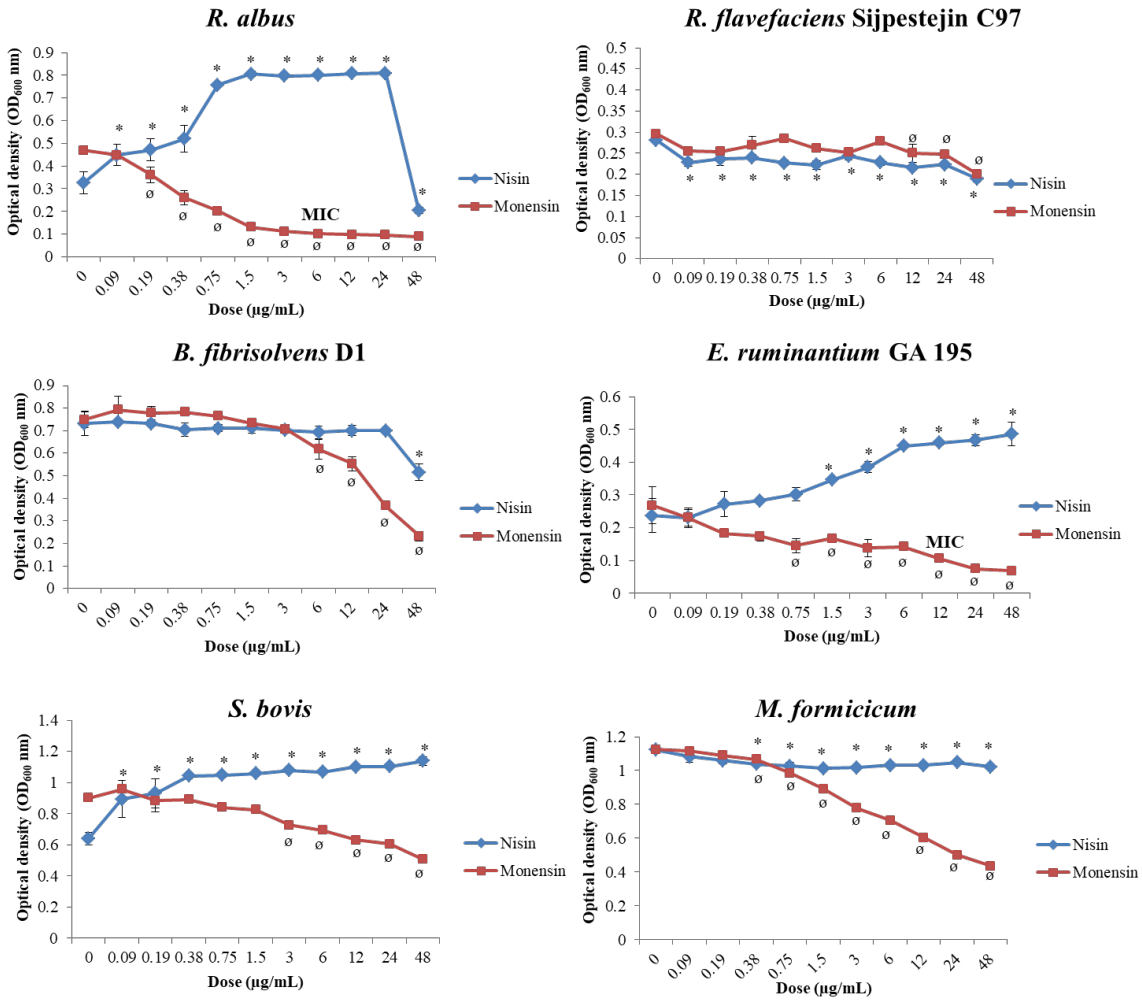

Figure 2: Effects of nisin and monensin on Gram-positive rumen bacteria. The results represent the mean ± standard error. *p<0.05, difference of the nisin-treated culture compared with the control; ºp<0.05, difference of the monensin-treated culture compared with the control. Control level was 0 µg/mL for both of nisin and monensin. MIC: Minimal inhibitory concentration

Şekil 2: *Nisin ve monensin'in Gram-pozitif rumen bakterileri üzerine etkileri. Sonuçlar ortalama ± standart hatayı temsil etmektedir. *p<0,05; nisin ile muamele edilmiş kültürün kontrol ile karşılaştırıldığında farkı; ºp<0,05; monensin ile muamele edilmiş kültürün kontrol ile karşılaştırıldığında farkı. Kontrol seviyesi hem nisin hem de monensin için 0 µg/mL'dir. MİK: Minimal inhibitor konsantrasyon*

Both nisin and monesin show growth stimulatory effect on Gram-negative rumen bacteria (p<0.05). Nisin and monensin promoted the growth of *M. elsdenii* at 1.5-48 µg/mL and 0.19-48 µg/mL doses, respectively (p<0.05). Nisin and monensin also showed stimulatory effect on *F. succinogenes* from 0.75 µg/mL (p<0.05). However, the stimulatory effect of nisin was higher and permanent from 3 µg/mL dose while the effect of monensin disappeared at 24-48 µg/mL.

4. Discussion and Conclusion

Antibiotic feed additives have been used since the 1970s to decrease fermentation losses and increase the useful end-products of ruminal fermentation (6). Ionophore antibiotics alter ruminal fermentation via affecting Gram-positive rumen bacteria while protecting Gram-negative ones (26). In this study, Gram-positive rumen bacteria were more sensitive to monensin than Gram-negatives, consistent with the literature. *R. albus* and *E. ruminantium* were the most sensitive bacteria to monensin. Growth of these bacteria was inhibited completely by monensin, at 6 and 12 µg/mL concentrations respectively. Monensin did not inhibit the other Gram-positive bacteria though it diminished the bacterial growth compared the control. The minimal inhibitory concentrations (MICs) of monensin on *R. flavefaciens* and *S. bovis* were higher than 40 µg/mL according to Slyter et al. (30), similar with the results of the present study. On the other hand, *R. albus*, *B. fibrisolvens*, and *S. bovis* were inhibited to differing extents by monensin at concentrations between 0.1 and 10 µg/mL (11). Dawson and Boling (9) reported that the MICs of monensin and lasalocid were greatly influenced by potassium concentrations in the medium. The MIC values of monensin and lasalocid on *Bacteroides ruminicola* were 16-64 times greater when the potassium concentration of the medium increases from 4.6 to 12.3 mM. Ionophore antibiotics exhibit antibacterial activity via depleting intracellar potassium and, antimicrobial activities of some ionophores can be reversed by increasing the potassium concentrations in the medium (9). Therefore, resistance of some Gram-positive bacteria to monensin might be related with the relatively high potassium concentration of the medium (approximately 12.8 mM) used in the present study.

Antibacterial effect of monensin on Gram-positive rumen bacteria was higher than nisin in the present study. Besides, surprisingly, nisin exhibited growth stimulatory effects on some Gram-positive bacteria like *R. albus*, *E. ruminantium,* and *S. bovis*. Literature on the effects of nisin on the pure cultures of rumen bacteria is scarce. Although nisin is known as primarily effective on Gram-positive bacteria (2), it is reported that some Gram-positive bacteria can become nisin resistant. According to Mantovani and Russell (18), any nisin-sensitive cell can become nisin resistant as long as the ratio of nisin to cells is not too high and the incubation period is long enough. In that study (18), nisin (1 mM) caused a decrease in the viability of *S. bovis* culture during the first hour of incubation, but the viable cell number started to increase from the second hour and continued to multiply until the end of 8-hour incubation. So, the *R. albus*, *E. ruminantium,* and *S. bovis* cultures seemed to become resistant to nisin during the 24 hours of incubation in the present study. However, the sharp drop in the optical density of *R. albus* culture at 48 µg/mL of nisin treatment suggested that the related dose was too high to allow bacterial resistance. Nisin exhibits antibacterial activity via aggregating to form a pore through the cell membrane and causing potassium loss from the cell (25). Nisin is a positive-charged protein (2). Researchers indicated that the nisin-resistant cells had an increased positive charge than nisin-sensitive cells therefore; they excluded some of the nisin (18). Resistant cells bound less cytochrome c –an apoptosis related protein- because of the same reason. Nisin-resistant cells were also more lysozyme resistant, and were less hydrophobic (18).

On the other hand, resistance development theory might be not sufficient to explain the growth stimulatory effects of nisin treatment on some Gram-positive bacteria in the present study. Kišidayová et al. (16) reported that the supplementation of nisin significantly increased the *in vitro* population of major ciliate groups, *Entodinium* spp. and *Dasytricha ruminantium*, in the rumen while monensin significantly decreased the population of both groups in the same study. It is known that some plant metabolites when used at low doses can be converted to more bioactive forms as a result of bacterial degradation and these end-products can stimulate synthesis of bacterial proteins (1, 23). A bacteriocin-like inhibitory substance (BLIS), which is produced by *B. fibrisolvens* JL5, was reported to be degraded with a bacterial enzyme, pronase E (27). Nisin might be inactivated with a similar mechanism that is in need of illumination.

The effects of nisin and monensin on Gram-negative rumen bacteria were in the same direction in the present study. *M. elsdenii* and *F. succinogenes* were resistant to both substances. Results of the present study are consistent with the reports indicate the resistance of Gram-negative bacteria to nisin (7) and monensin (11). Gram-negative bacteria, unlike Gram-positive bacteria, have an outer membrane which protects the cell membrane from antimicrobial substances (4). The growth of Gram-negative bacteria was also promoted moderately by nisin and

monensin and, the stimulatory effect was more evident and permanent with nisin in the present study. The stimulatory effects of nisin on both Gram-negative and some Gram-positive rumen bacteria might be the explanation of the increase on ruminal acetate and propionate production with nisin treatment, unlike monensin, which increased only propionate production (13). In contrast to monensin, the lack of an adverse effect of nisin on dry matter digestibility (29) might also be a result of nisin's non-inhibitory effect on ruminal bacteria such as *R. albus*, *E. ruminantium,* and *F. succinogenes* that play key roles in cellulose digestion.

As a conclusion, the different effects of nisin and monensin particularly on some Gram-positive rumen bacteria probably responsible for their inconsistent effects on ruminal fermentation. Future *in vitro* and *in vivo* studies are needed to elucidate the nisin's mode of action on rumen bacteria, and validate the efficiency of nisin to modify bacterial profile in the rumen.

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