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Abstract

Nisin, a well-established bacteriocin utilized in the food industry, has been recognized as a potential substitute for monensin in livestock applications. This study aims to evaluate and compare the effects of nisin and monensin supplementation on ruminal and colonic microbiota and their fermentation profiles in fattening Hu lambs. Thirty-six male Hu lambs (23.5 ± 1.0 kg) were assigned to one of three dietary treatments using a randomized block design. The treatments included: (1) a control group receiving a basal diet (CON); (2) the basal diet supplemented with 40 mg/kg dry matter (DM) of monensin (MON); and (3) the basal diet supplemented with 274.5 mg/kg DM of a nisin (NIS). The trial lasted for 9 weeks. At the end of the experiment, one sheep from each pen (18 sheep in total) was randomly selected for humane slaughter, and rumen and colon content samples were collected for the analysis of fermentation products and microbiota composition. The inclusion of NIS and MON did not impact the final slaughter performance or rumen development in Hu lambs. NIS supplementation resulted in a decrease in the populations of total bacteria, fungi, and methanogens, while simultaneously increasing the abundance of specific fiber-degrading bacteria, such as those within the family *Ruminococcaceae*, compared to the CON group. However, NIS did not alter the structure or abundance of individual bacterial taxa in the colon. While NIS showed no notable effect on rumen fermentation, it exerted a marked influence on fermentation in the colon. Conversely, MON led to a reduction in the abundance of Gram-positive bacteria, such as Firmicutes, while increasing the prevalence of Gram-negative bacteria, including Bacteroidetes and Proteobacteria, in the rumen; these effects were even more pronounced in the colon. As a result, the addition of MON influenced fermentation processes in both the rumen and the colon. In conclusion, NIS supplementation significantly impacted the rumen microbiota and colonic fermentation in Hu lambs, with its primary effects observed in the rumen. In contrast, MON influenced both the rumen and colonic bacterial communities, demonstrating a notably more pronounced effect on the colonic microbiota.

Keywords: Nisin, Monensin, Fattening Hu lambs, Rumen and Colon Fermentation, Microbiota

INTRODUCTION

The global ban of antibiotics as growth promoters in animal feeding operations is primarily driven by concerns over antibiotic residues and the rise of bacterial resistance, which significantly impacts livestock production. This has created an urgent need to explore viable alternatives. Bacteriocins, a class of natural antimicrobial proteins ribosomally synthesized by bacteria, have emerged as a promising alternative. These proteins exert their antimicrobial effects by disrupting cell membranes or inhibiting nucleic acid replication, thereby suppressing susceptible bacteria [1]. Unlike traditional antibiotics, bacteriocins offer several advantages, including a lower risk of resistance development, lack of harmful residues, and greater environmental safety, making them an attractive option for livestock production [2]. Nisin (NIS) is one of the most extensively studied bacteriocins. It is an antimicrobial peptide produced by *Lactococcus* and *Streptococcus* species [3], and it is particularly effective against Gram-positive bacteria, including the inhibition of spore-forming *Bacillus* and *Clostridium* species. Due to its potent antimicrobial properties, NIS has been widely utilized in the food industry to control pathogenic bacteria [4]. Although concerns have been raised regarding its stability in the gastrointestinal (GI) tract, Lee et al. (2002) [5] demonstrated that NIS primarily associates with rumen microbes rather than undergoing degradation, highlighting its potential as a modulator of the rumen microbiota. Recent studies have further underscored the significant promise of NIS as an antibiotic alternative in livestock production [6–8].

Indeed, a recent study showed that NIS alleviates the adverse effects of *Clostridium perfringens* on growth performance and intestinal integrity in broiler chickens by reducing inflammation and intestinal apoptosis and modulating the cecum microbiota [9]. Similarly, O'Reilly et al. (2023) [10] demonstrated NIS's ability to modulate the GI microbiota and influence its function in a pig model. Limited research has explored the effects of NIS on ruminant production, particularly *in vivo*. *In vitro* studies suggest that NIS and monensin (MON), a widely used ionophore antibiotic, differentially affect bacterial populations and communities, yet both can reduce methane production and modulate rumen fermentation patterns, while NIS may be more effective than MON in optimizing ruminal microbial ecology and reducing methane emissions [11,12]. Given the inherent differences between *in vitro* and *in vivo* conditions in terms of substrate availability and microbial diversity [13], we conducted an *in vivo* experiment to evaluate NIS and MON supplementation in Hu sheep [14]. Our preliminary findings revealed that both additives effectively reduced ruminal acetate concentrations and the acetate-to-propionate ratio, suggesting a

shift in fermentation patterns potentially linked to microbiota modulation. Based on these observations, we hypothesized that NIS and MON exert distinct effects on the rumen microbiota *in vivo*.

Beyond the rumen, the hindgut plays a critical role in fiber fermentation, contributing additional energy and nutrients to the host. It is estimated that hindgut fermentation accounts for approximately 14% of total rumen fermentation capacity [15] and supplies 8%–17% of the total volatile fatty acids (VFAs) absorbed in the ruminant GI tract [16]. The colon, a key component of the hindgut, facilitates water absorption, waste elimination, and electrolyte homeostasis while supporting microbial fermentation of undigested plant material. The resulting VFAs, such as acetate and propionate, serve as energy sources and influence nutrient utilization and growth performance [15,17]. While ruminal bacteria [5] and trypsin activity [18] can degrade NIS, potentially limiting its impact on colonic fermentation, MON is known to persist in the GI tract, with approximately 50% remaining unmetabolized and accumulating in the colon [19]. This suggests that MON may exert a more pronounced effect on colonic fermentation and microbiota composition than NIS. In this study, we utilized fattening Hu lambs as an animal model to comparatively evaluate the effects of dietary NIS and MON supplementation on rumen and colon fermentation and microbiota using metataxonomics. The findings provide a theoretical basis for considering NIS as a potential alternative to MON in ruminant nutrition and production.

MATERIALS AND METHODS

Animals, diets, and experimental design

The experimental protocol was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Protocol number: SYXK2017-0007). The study followed a randomized complete block design and was conducted according to the methodology described in a previous study [14].

Briefly, 36 male Hu lambs (23.5 ± 1.0 kg) were stratified into two body weight (BW) blocks (low and high) and randomly assigned within each block to one of three dietary treatments. The treatments included (i) a control (CON) group receiving a basal diet without additives, (ii) a monensin (MON) group supplemented with 40 mg MON/kg dry matter (DM), and (iii) a nisin (NIS) group supplemented with 274.5 mg NIS/kg DM. Nisin (2.5% purity; $\geq 1 \times 10^6$ IU/g) was sourced from Zhejiang New Yinxiang Bioengineering Co., Ltd, while monensin premix (20% MON sodium) was obtained from Shandong Shengli Bioengineering Co., Ltd.

The basal diet was formulated to meet the Feeding Standard for Meat-Producing Sheep (Ministry of Agriculture, China, 2004; Table 1) and was offered as a total mixed ration (TMR), with MON or NIS thoroughly mixed in before feeding. Lambs were fed twice daily (08:00 and 16:00) with feed provided at a 5-10% feed refusal allowance. The study lasted nine weeks, consisting of a two-week adaptation period followed by seven weeks of dietary intervention. All lambs were housed in indoor pens (2 m × 4 m) with wooden slatted floors, with two lambs per pen, and had unrestricted access to water.

Sampling and measurement

Slaughter performance and rumen development indicators

At the end of the trial, one lamb per pen (n = 18) was randomly selected for slaughter. Lambs were weighed pre-slaughter, and the carcass was harvested and weighed immediately. The liver, rumen, and colon were excised and weighed. Rumen and colon contents were collected for fermentation parameter analysis and DNA extraction. The rumen epithelium was rinsed with saline, and its cleaned weight was recorded. Organ indices were calculated as follows:

$$\text{Organ index (\%)} = \text{organ weight} / \text{carcass weight} \times 100\%$$

Rumen epithelial samples from the abdominal blind sac were collected for papilla measurements following Walton et al. (2002) [20]. The samples were first rinsed with phosphate-buffered saline (PBS), and a 1-cm^2 section was then excised. The number of papillae per section was counted, and their length and width were measured using vernier calipers. Five distinct regions were chosen for each sample to ensure precision, with three rumen papillae measured within each region. The rumen papilla area was calculated as papilla length × papilla width × 2.

The carcasses were weighed to determine the slaughter rate, calculated as follows: Slaughter rate (%) = carcass weight / pre-slaughter weight × 100%.

Rumen and Colon Fermentation Parameters

The pH of rumen and colon chyme samples was measured immediately after collection using a pH meter. For VFA analysis, 0.2 mL of 25% metaphosphoric acid was added to 1 mL of rumen fluid and a 1:10 dilution of colon chyme. VFAs were quantified using gas chromatography (7890A, Agilent, UK) following the method of Mao et al. (2008) [21]. Ammonia nitrogen (NH_3-N) concentration was measured colorimetrically [22].

Microbiota analysis

DNA extraction

Metagenomic DNA was extracted from the rumen and colon chyme samples using the bead-beating and phenol-chloroform method [23]. DNA quality was assessed via agarose gel (1.2%) electrophoresis, and concentration was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher, USA), with the A_{260}/A_{280} ratio calculated using the absorbance at 260 and 280 nm. Samples were stored at −20°C until analysis.

Real-time PCR quantification of microbial populations

Select microbial populations, including total bacteria (targeting the 16S rRNA gene), fungi (targeting the 18S rRNA gene), protozoa (targeting the 18S rRNA gene), and methanogens (targeting the *mcr*A gene), were quantified by real-time PCR following Shen et al. (2017) [11]. Briefly, each reaction (20 μL) consisted of 10 μL of TB GREEN, 0.4 μL of ROX, 0.4 μL of each forward and reverse primer (Supplementary Table S1), 6.8 μL of double-distilled water, and 2 μL of DNA template. PCR was performed using an ABI 7500 Real-Time PCR instrument (Applied Biosystems, USA). Triplicate reactions were conducted per sample, with absolute abundance, expressed as the number of copies of the target gene per mL of the sample, determined from standard curves generated using serially diluted plasmid DNA containing the target sequence.

Metataxonomic analysis of bacterial and archaeal communities

Individual amplicon sequencing libraries were prepared using PCR targeting the V3-V4 hypervariable region of the 16S rRNA gene with primers 338F (5'-ACTCCTACGGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') [11], each with a Junction Barcode (8 bases). The expected PCR band was visualized using agarose gel (2%) electrophoresis and then excised and purified using the AxyPrepDNA Gel Extraction Kit (Axygen Biosciences, USA). The purified amplicon libraries were quantified concentrations using the QuantiFluor® dsDNA kit (Promega, USA). Then, the amplicon libraries were pooled in equal proportions and paired-end (2 x 300 bp) sequenced on the Illumina MiSeq platform.

Raw amplicon sequences were analyzed using QIIME version 1.9.1, as previously described [25]. Briefly, after demultiplexing, the barcode and primer sequences were trimmed off. Following quality filtering and read merging, the sequences were grouped into operational taxonomic units (OTUs) at 97% sequence identity using UPARSE (version 7.1), and possible chimeric sequences were identified and removed using the UCHIME algorithm [26]. Subsequently, the most predominant sequence was selected as the representative sequence, which

was taxonomically classified by comparison to the SILVA database version 138 [27]. Alpha diversity metrics, including OTU count, coverage, Chao 1 richness estimate, and Shannon diversity index, were computed. Beta diversity was analyzed using Mothur based on the Bray-Curtis dissimilarity [27].

Statistical Analyses

The microbial alpha-diversity and relative abundance data were analyzed at the phylum and genus levels using Kruskal–Wallis non-parametric tests with the SPSS software. Fermentation parameters were analyzed for significant differences between treatments and the two GI sites using Tukey's test following one-way ANOVA in SPSS software. Beta diversity of the rumen and colon microbiotas was analyzed using principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity, and significance was assessed using Analysis of Similarity (ANOSIM). Statistical significance was defined as highly significant ($p < 0.01$), significant ($p < 0.05$), and a trend ($0.05 < p < 0.1$). Correlations between fermentation parameters and the relative abundance of bacterial genera were assessed using the Bioinformatics Cloud Platform (Shenzhen Microbiota Technology Co. Ltd., website: www.bioincloud.tech).

RESULTS

Growth performance and slaughter performance

Neither NIS nor monensin MON supplementation significantly affected ($p > 0.05$) dry matter intake (DMI), growth performance (Supplemental Table S2), carcass weight, liver weight, rumen dry/wet weight, colon wet weight, rumen papilla length, or rumen papilla width in fattening Hu lambs compared to the CON group (Table 2). However, MON supplementation showed a decreasing trend ($p = 0.09$) in rumen papillae density and area relative to the CON and NIS groups.

Rumen and colon fermentation parameters

MON supplementation significantly increased ($p < 0.05$) rumen pH while decreasing ($p < 0.05$) the concentrations of NH_3-N, total VFA, acetate, butyrate, and valerate compared to the CON group (Table 3). In contrast, NIS did not significantly alter ($p > 0.05$) rumen fermentation parameters. Additionally, at the VFA composition, MON significantly increased ($p < 0.05$) the propionate proportion while decreasing ($p < 0.05$) the butyrate and valeric acid proportions compared to the CON group.

Additionally, MON increased ($p < 0.05$) colon pH relative to the CON group. Both NIS and MON significantly reduced ($p < 0.05$) colon total VFA and acetate levels, but they exhibited distinct effects on colon propionate concentration: NIS decreased propionate, whereas MON increased it ($p < 0.05$). Furthermore, NIS significantly reduced ($p < 0.05$) NH_3-N concentration in the colon. Among VFA composition, MON significantly reduced ($p < 0.01$) the proportion of acetate production and significantly increased ($p < 0.01$) the proportion of propionate production.

Rumen and colon microbial populations

Compared to the CON group, NIS significantly decreased ($p < 0.05$) the populations of total bacteria, fungi, and methanogens in the rumen, while MON had no effect ($p > 0.05$) on these microbial populations (Fig. 1A). In the colon, neither NIS nor MON affected ($p > 0.05$) the populations of fungi, protozoa, or methanogens. However, MON significantly decreased ($p < 0.05$) the total bacterial population in the colon (Fig. 1B).

Rumen and colon bacterial diversity

Sequencing depth coverage exceeded 99% across all treatment groups, indicating adequate sequencing depth (Table 4). Neither MON nor NIS affected ($p > 0.05$) alpha diversity metrics in the rumen. Similarly, NIS had no significant impact ($p > 0.05$) on colon bacterial alpha diversity. However, MON supplementation significantly decreased ($p < 0.05$) all-alpha diversity metrics in the colon, suggesting a negative effect on colon bacterial diversity. PCoA revealed significant differences in ruminal bacterial community structures among the three groups (ANOSIM, $R = 0.33$, $p < 0.001$). Additionally, the colonic bacterial community structures significantly differed between the MON and CON groups ($R = 0.56$, $p < 0.001$; Fig. 2B).

Rumen and colon bacterial composition at the phylum level

In the rumen, 23 bacterial phyla were identified, with *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Spirochaetes* being predominant. *Bacteroidetes* (47.54-49.86%) and *Firmicutes* (43.00-45.79%) comprised the majority (>92.86%) of the bacterial population (Fig. 3A). NIS did not significantly alter ($p > 0.05$) the relative abundance of any phylum, whereas MON significantly increased ($p < 0.05$) the relative abundance of *Proteobacteria*.

In the colon, 20 bacterial phyla were identified, with *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* being predominant. Similar to the observations in the rumen, *Firmicutes* (62.16-83.64%) and

Bacteroidetes (10.77-24.66%) were the most predominant phyla (Fig. 3B). NIS did not affect ($p > 0.05$) colonic bacterial phyla, while MON significantly decreased ($p < 0.05$) the relative abundance of *Firmicutes* and increased ($p < 0.05$) the relative abundance of *Bacteroidetes*, *Actinobacteria*, and *Patescibacteria*.

Rumen and colon bacterial composition at the genus level

Across all treatment groups, 50 bacterial genera were identified in the rumen, with 17 genera exhibiting relative abundances greater than 1% (Fig. 4A). The most abundant genera included *Prevotella* (29.92–31.22%), the Christensenellaceae R-7 group (7.87–11.03%), and the Ruminococcaceae NK4A214 group (4.30–7.12%), collectively accounting for 44.89% of the total rumen bacterial population. NIS supplementation significantly increased ($p < 0.05$) the relative abundance of the Ruminococcaceae NK4A214 group and Lachnospiraceae uncultured, whereas MON had no significant effect ($p > 0.05$) on rumen bacterial composition at the genus level.

In the colon, 50 bacterial genera were identified, with 33 genera exceeding 1% relative abundance (Fig. 4C). Ruminococcaceae UCG-005 (2.60–16.23%) and the Christensenellaceae R-7 group (6.57–14.69%) were the most prevalent. NIS did not affect ($p > 0.05$) the relative abundance of any colonic bacterial genera. However, MON significantly altered ($p < 0.05$) the relative abundance of multiple genera, including reductions in Ruminococcaceae UCG-005, the Eubacterium coprostanoligenes group, Ruminococcaceae UCG-010, the Lachnospiraceae NK4A136 group, and increases in Muribaculaceae norank, the Lachnospiraceae NK3A20 group, and Bifidobacteriaceae Unclassified.

Correlations between Fermentation Parameters and Dominant Bacterial Genera

Correlation analysis indicated that *Prevotella* exhibited a negative correlation with rumen pH ($p < 0.05$; Fig. 5A). The group F082 norank exhibited a positive correlation with rumen pH but a negative correlation with butyrate and valerate concentrations ($p < 0.05$). The Ruminococcaceae NK4A214 group and Lachnospiraceae uncultured were positively correlated with NH_3-N concentration ($p < 0.05$). Additionally, the Rikenellaceae RC9 gut group correlated positively with isovalerate concentration ($p < 0.05$), while *Muribaculaceae* norank was negatively correlated with valerate concentration ($p < 0.05$). *Saccharofermentans* exhibited a negative correlation with acetate concentration ($p < 0.05$), whereas Ruminococcus-2 was negatively correlated with isobutyrate, isovalerate, and total branched-chain VFAs ($p < 0.05$).

In the colon, Ruminococcaceae UCG-005, the Ruminococcaceae NK4A214 group, and the Ruminococcaceae NK4A136 group were negatively correlated with propionate concentration but positively correlated with the acetate-to-propionate ratio ($p < 0.05$) (Fig. 5B). Several genera, including Eubacterium coprostanoligenes, Ruminococcaceae uncultured, the Rikenellaceae RC9 gut group, and Ruminococcaceae UCG-013, were negatively correlated with propionate and butyrate concentrations ($p < 0.05$). Additionally, Muribaculaceae norank and Bifidobacteriaceae-Unclassified displayed a negative correlation with the acetate-to-propionate ratio, whereas Tyzzerella 4 showed a positive correlation ($p < 0.05$). Ruminococcaceae UCG-010 negatively correlated with the concentrations of NH_3-N, propionate, and butyrate ($p < 0.05$), while Ruminococcus 2 showed a negative correlation with isovalerate concentration, and Ruminococcaceae UCG-014 displayed a positive correlation ($p < 0.05$). Significant correlations ($p < 0.05$) were also observed for other genera, including a positive correlation between propionate concentration and *Blautia*, *Succinivibrio*, and the Lachnospiraceae NK3A20 group, a positive correlation between *Roseburia* and total VFA, acetate, and butyrate concentrations, a negative correlation between Prevotella 9 and valerate concentration, a positive correlation between propionate concentration and *Bacteroides* and *Succiniciasticum*, a positive correlation between the Ruminococcus gauvreauii group and propionate concentration, but a negative correlation with isovalerate concentration ($p < 0.05$).

DISCUSSION

This study builds upon our previous research, including both *in vitro* and *in vivo* experiments [11, 14], to comparatively evaluate the effects of NIS and MON on rumen and colonic fermentation, microbiota composition, and feed digestion in fattening Hu lambs. As hypothesized, NIS and MON exerted distinct influences on rumen and colonic fermentation and microbiota. NIS primarily affected the rumen microbiota, whereas MON altered the microbiota in both the rumen and colon, with more pronounced effects in the latter.

Effect on rumen fermentation and microbiota

The rumen epithelium, especially the structure of rumen papillae, plays a crucial role in nutrient absorption, particularly VFA absorption, and feed efficiency [28]. Our findings revealed that MON supplementation resulted in the smallest rumen papillae area and density, whereas NIS promoted the largest papillae area. This suggests that NIS may enhance ruminal epithelial development and nutrient absorption, while MON may inhibit this process.

Rumen bacteria are central to the function of the rumen ecosystem, influencing host nutrition and health [29]. Consistent with a prior *in vitro* study [12], NIS supplementation reduced the total bacterial, fungal, and methanogen populations but did not significantly alter rumen fermentation parameters, which aligns with previous *in vivo* studies [30]. Feed digestion and rumen fermentation rate depend on microbial diversity and abundance, which in turn influence metabolic capacity and environmental stability [31]. Indeed, high microbial diversity is associated with an improved ability to digest high-fiber forage in the rumen of high-plateau yaks [32]. Despite reducing specific microbial populations, NIS did not affect overall bacterial richness or diversity, likely explaining the absence of significant changes in fermentation parameters and nutrient digestion, as reported by Luo et al. (2024) [14]. Although nisin is known for its selective inhibition of gram-positive bacteria in food industry applications, our results showed a general suppression of total bacteria and even an increase in some gram-positive bacteria, such as the Ruminococcaceae NK4A214 group and Lachnospiraceae_uncultured. This discrepancy may be due to the complex rumen environment, where microbial interactions and host factors influence the response to nisin.

NIS reduced the acetate concentration while increasing that of propionate, leading to a lower acetate-to-propionate ratio, which is consistent with previous in vitro studies [11,12]. Since propionate production is negatively correlated with methane emissions, this shift suggests a potential methane-reducing effect of NIS. This is further supported by the observed reduction in methanogen and fungal populations and an increase in a higher proportion of propionate production [12]. Although methane production was not directly measured, these findings imply that NIS may mitigate methane emissions by redirecting hydrogen from methanogenesis to propionate synthesis. Additionally, the positive correlation between ammonia-nitrogen concentration and fiber-degrading bacteria suggests that NIS may enhance the synchronization of carbon and nitrogen release, thereby improving energy utilization efficiency. However, further investigations are required to confirm this premise.

Compared to CON and NIS, MON supplementation decreased total VFA concentrations in the rumen without significantly affecting the final carcass weight. This could be attributed to compensatory metabolic pathways in the animals that maintain energy supply despite reduced VFA supply [33, 34]. The decline in acetate concentration and the increase in propionate concentration resulted in a lower acetate-to-propionate ratio, consistent with previous findings [11, 35]. Since propionate production is associated with more efficient energy utilization and lower methane emissions than acetate and butyrate [36], it is widely recognized that MON contributes to methane

reduction by reducing hydrogen production associated with acetate fermentation rather than directly inhibiting methanogens [35, 37]. This is further corroborated in the present study, which showed no changes in methanogen abundance but decreased relative abundance of hydrogen-producing bacteria, such as members of Firmicutes, the primary phylum containing major rumen H_2-producing bacteria. Additionally, the observed decrease in ruminal NH_3-N concentration by MON is likely due to its inhibition of amino acid deamination, as reported in previous studies [38, 39].

Effects on colon fermentation and microbiota

As a peptide, NIS can be degraded by rumen bacteria [5] and host tryptase [16, 40], leaving little dietary NIS to reach the colon. This may explain the minimal impact of NIS on the colonic bacterial community. However, despite the lack of substantial effects on colonic bacteria at the phylum or the genus level, NIS significantly altered some colonic fermentation parameters by decreasing total VFA, propionate, and ammonia. This discrepancy suggests that the changes in colonic fermentation may stem from modifications in the nutrient composition of the chyme entering the colon rather than from direct microbial modulation. In other words, by influencing foregut microbiota and function, NIS could modify the substrates available for hindgut fermentation, subsequently impacting the profile of colonic fermentation products.

Unlike NIS, MON is an ionophore, with approximately half of the administered dose remaining unabsorbed and unmetabolized in cattle or sheep [19], allowing it to reach the large intestine. Additionally, limited evidence supports MON being degraded by rumen bacteria, suggesting that MON may accumulate in the large intestine. This accumulation may explain the alterations in the colonic bacterial community observed in the MON group. MON had a more profound impact on the colon than on the rumen in terms of altering the composition and abundance of the bacterial community. Further analyses revealed a consistent effect of MON on the bacterial community in both the colon and the rumen, notably suppressing Gram-positive bacteria such as members of Firmicutes, including some specific taxa like Ruminococcaceae UCG-005 and Ruminococcaceae UCG-010, while promoting Gram-negative bacteria like those within Bacteroidetes. Since Firmicutes bacteria can degrade carbohydrates [41, 42], and certain Gram-positive fiber-degrading bacteria within this phylum, such as *Ruminococcus* spp., are major acetate producers [43], their suppression by MON likely contributed to the reduced acetate and increased propionate observed in the colon. The negative relationship between *Ruminococcus* spp. and

the levels of propionate and butyrate in the colon corroborates this premise. These findings suggest that MON can modulate colonic fermentation by altering bacterial composition, reducing acetate production, and increasing propionate and butyrate production. These effects may lead to reduced methane emissions in the colon of Hu lambs.

It is noteworthy that the antibacterial effect of MON in the hindgut suggests that there remained some residual MON in the GI tract, which may ultimately be excreted via the feces, as previously reported in a study [44]. When the antibiotics-containing faces enter the soil, they may trigger the accumulation of antibiotic resistance genes (ARGs), thereby posing a food safety risk. A recent study showed that MON treatment could lead to an increase in ARGs, which may migrate from feces to cultivated mushrooms [45]. In contrast, NIS may have a superior safety profile because it undergoes continuous degradation by microorganisms and enzymes in the GI tract, and it likely has no residual issues. Future studies may use metagenomic sequencing and mobile genetic element (MGE) analysis to systematically evaluate the effects of both on the abundance and potential spread of ARGs in the GI microbiome, further exploring the feasibility and safety of NIS as an alternative to MON.

CONCLUSION

Dietary supplementation with MON significantly altered both ruminal and colonic microbial communities in fattening Hu lambs, with a more pronounced impact within the colon. MON selectively inhibited Gram-positive bacteria in both regions, thereby modifying fermentation patterns. In contrast, NIS primarily influenced the rumen, reducing total bacterial, fungal, and methanogen populations while maintaining overall fermentation efficiency and carcass performance. These findings highlight the distinct mechanisms by which NIS and MON modulate the ruminal and colonic microbiota, with potential implications for methane mitigation and feed efficiency in ruminant production systems.

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436 Table 1. Ingredient and nutrient composition of the experimental diets

Item	Basal diet
Ingredient (% of DM)	
Corn silage	23.00
Peanut vine	22.00
Corn grain	30.00
Soybean meal	7.00
DDGS	12.00
Wheat bran	2.00
Urea	0.75
Salt	0.75
Sodium bicarbonate	1.00
Calcium carbonate	1.00
Calcium monophosphate	0.37
Mineral Premix[1]	0.10
Vitamin Premix[2]	0.03
Nutrient composition (% of DM)	
DM	49.7
CP	15.71
NDF	30.38
ADF	17.92
EE	3.51
Ash	9.48
DE, MJ/kg	13.25

437 [1]Formulated to provide (per kilogram of DM): Cu, 18, 000 mg; Fe, 35, 500 mg; Co, 300 mg; I, 1, 650 mg; Se,

438 340 mg; Zn, 42, 000 mg; Mn, 36, 000 mg. [2]Formulated to provide (per gram of DM): vitamin A, 20, 040 IU;

439 vitamin D_3, 6, 600 IU; vitamin E, 200 IU.

Table 2. Effects of nisin and monensin on carcass performance and rumen development index in fattening Hu lambs

Item	CON	MON	NIS	SEM	*p*-value
Carcass weight, kg	17.78	18.23	18.35	0.188	0.46
Dressing percentage, %	50.75	49.87	51.28	2.102	0.53
Organ relative weight					
Liver weight, %	1.75	1.64	1.68	0.021	0.08
Colon fresh weight, %	2.40	2.37	2.24	0.069	0.61
Rumen Fresh weight, %	13.59	13.86	14.96	0.913	0.83
Rumen weight, %	1.63	1.74	1.63	0.037	0.39
Rumen papillae					
Length, mm	5.07	4.87	5.58	0.230	0.45
Width, mm	2.27	2.41	2.49	0.082	0.57
Number per cm^2	61.39	51.78	59.72	1.949	0.09
Area per cm^2	1400.22	1198.87	1649.10	85.438	0.09

CON, control (no additives); MON, 40 mg of monensin/DM kg of feed; NIS, 274.5 mg of nisin/DM kg of feed; SEM, standard error of means.

444 Table 3. Effects of nisin and monensin on rumen and colon fermentation parameters in fattening Hu lambs

445 [a-c] Means with different superscripts within a row differ ($P < 0.05$)

446

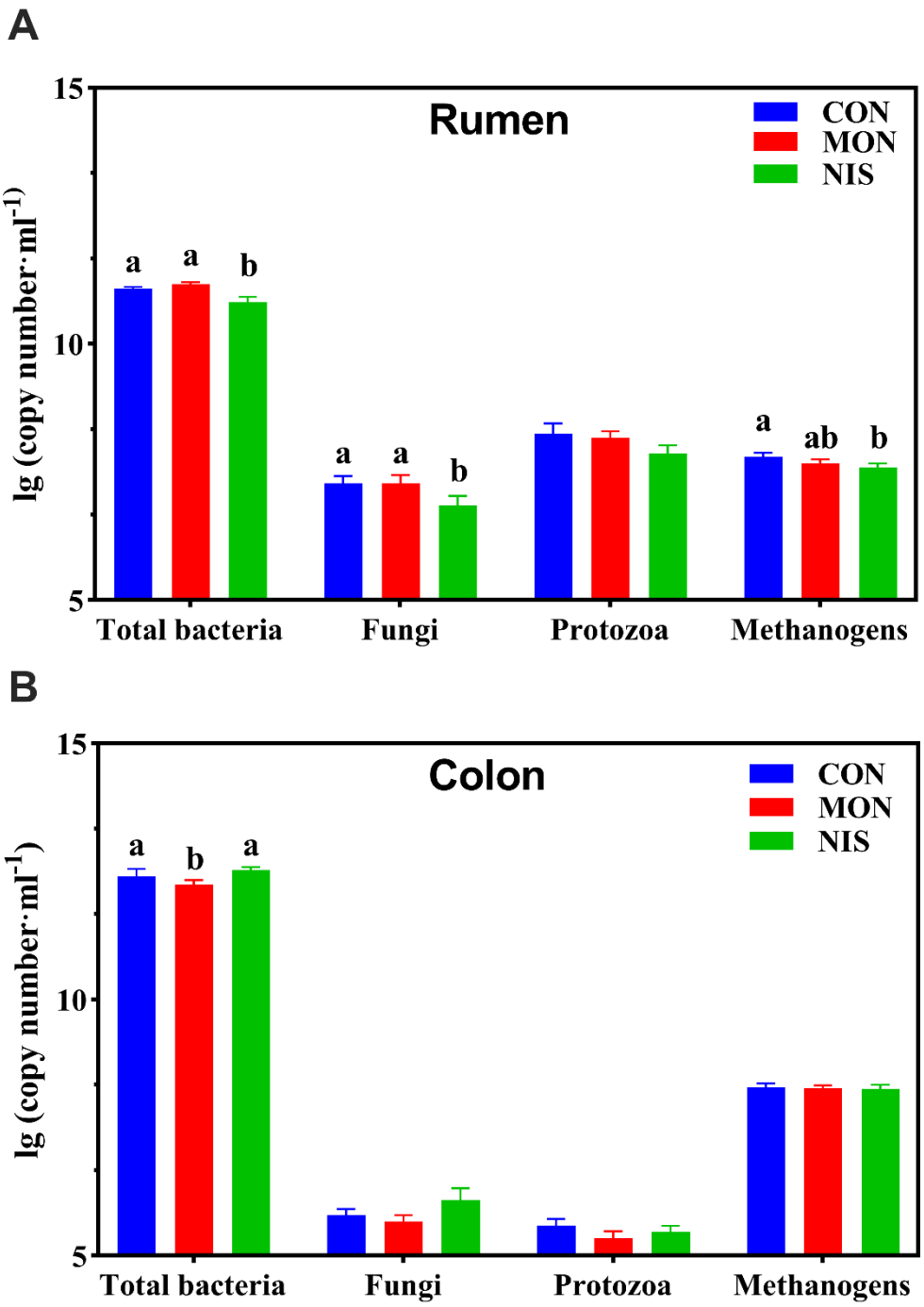
Item	CON	MON	NIS	SEM	*p*-value
Rumen					
pH value	5.84[b]	6.11[a]	5.91[b]	0.042	0.02
Ammonia, mg/dL	22.03[a]	15.97[b]	23.81[a]	1.351	0.03
Total VFA, mM	119.92[a]	106.80[b]	117.11[a]	2.249	0.03
Acetate, mM	75.58[a]	66.12[b]	72.02[a]	1.429	0.01
Propionate, mM	24.82	25.67	26.52	0.821	0.72
A: P	3.03[a]	2.60[b]	2.74[ab]	0.713	0.048
Butyrate, mM	13.63[a]	11.24[b]	14.64[a]	0.524	0.01
Valerate, mM	1.42[a]	1.10[b]	1.47[a]	0.059	<0.01
Isobutyrate, mM	0.83	0.88	0.84	0.039	0.88
Isovalerate, mM	1.53	1.80	1.62	0.120	0.68
Total BCVFA, mM	2.37	2.68	2.46	0.154	0.73
VFA composition，%					
Acetate	63.39	61.93	61.53	1.095	0.23
Propionate	20.81[b]	24.03[a]	22.6[ab]	1.014	0.02
Butyrate	11.95[a]	10.52[b]	12.52[a]	0.628	0.02
Valerate	1.18[a]	1.02[b]	1.26[a]	0.087	0.04
Colon					
pH value	6.70[b]	6.89[a]	6.73[ab]	0.035	0.049
Ammonia, mg/dL	17.80[a]	19.16[a]	14.09[b]	0.825	0.02
Total VFA, mM	71.35[a]	59.26[b]	56.39[b]	2.586	0.03
Acetate, mM	50.86[a]	36.88[b]	40.15[b]	2.064	<0.01
Propionate, mM	10.31[b]	12.46[a]	8.25[c]	0.547	<0.01
Butyrate, mM	7.39	7.76	5.39	0.635	0.27
Valerate, mM	1.10	0.92	0.94	0.039	0.12
Isobutyrate, mM	0.78	0.81	0.83	0.041	0.91
Isovalerate, mM	0.91	0.85	0.83	0.046	0.80
Total BCVFA, mM	1.69	1.66	1.66	0.083	0.99
VFA composition，%					
Acetate	71.49[a]	62.21[b]	71.1[a]	2.07	<0.01
Propionate	14.55[b]	20.33[a]	14.85[b]	1.375	<0.01
Butyrate	9.97	13.08	9.36	1.798	0.12
Valerate	1.56	1.55	1.68	0.155	0.63

Table 4. Effects of nisin and monensin on alpha diversity metrics of ruminal and colonic bacterial communities in fattening Hu lambs

Item	CON	MON	NIS	SEM	*p*-value
Rumen					
Coverage, %	98.81	98.88	98.78	0.081	0.93
OTUs	1899.7	1843.3	1702.0	37.85	0.24
ACE	2291.8	2238.0	2092.7	39.53	0.23
Chao1	2270.2	2230.8	2083.5	38.16	0.37
Shannon	5.65	5.71	5.72	0.082	0.75
Simpson	0.03	0.01	0.01	0.004	0.72
Colon					
Coverage, %	98.80	99.22	99.07	0.084	0.26
OTUs	1661.0^{a}	1217.8^{b}	1743.3^{a}	72.23	<0.01
ACE	2017.3^{a}	1477.7^{b}	2085.8^{a}	84.58	<0.01
Chao1	2018.5^{a}	1460.0^{b}	2059.7^{a}	83.46	<0.01
Shannon	5.88^{a}	4.97^{b}	5.76^{a}	0.117	<0.01
Simpson	0.01^{b}	0.03^{a}	0.01^{b}	0.004	<0.01

OTUs = operational taxonomic units; ACE = abundance-based coverage estimator.

$^{a\text{-}c}$ Means with different superscripts within a row differ ($P < 0.05$).



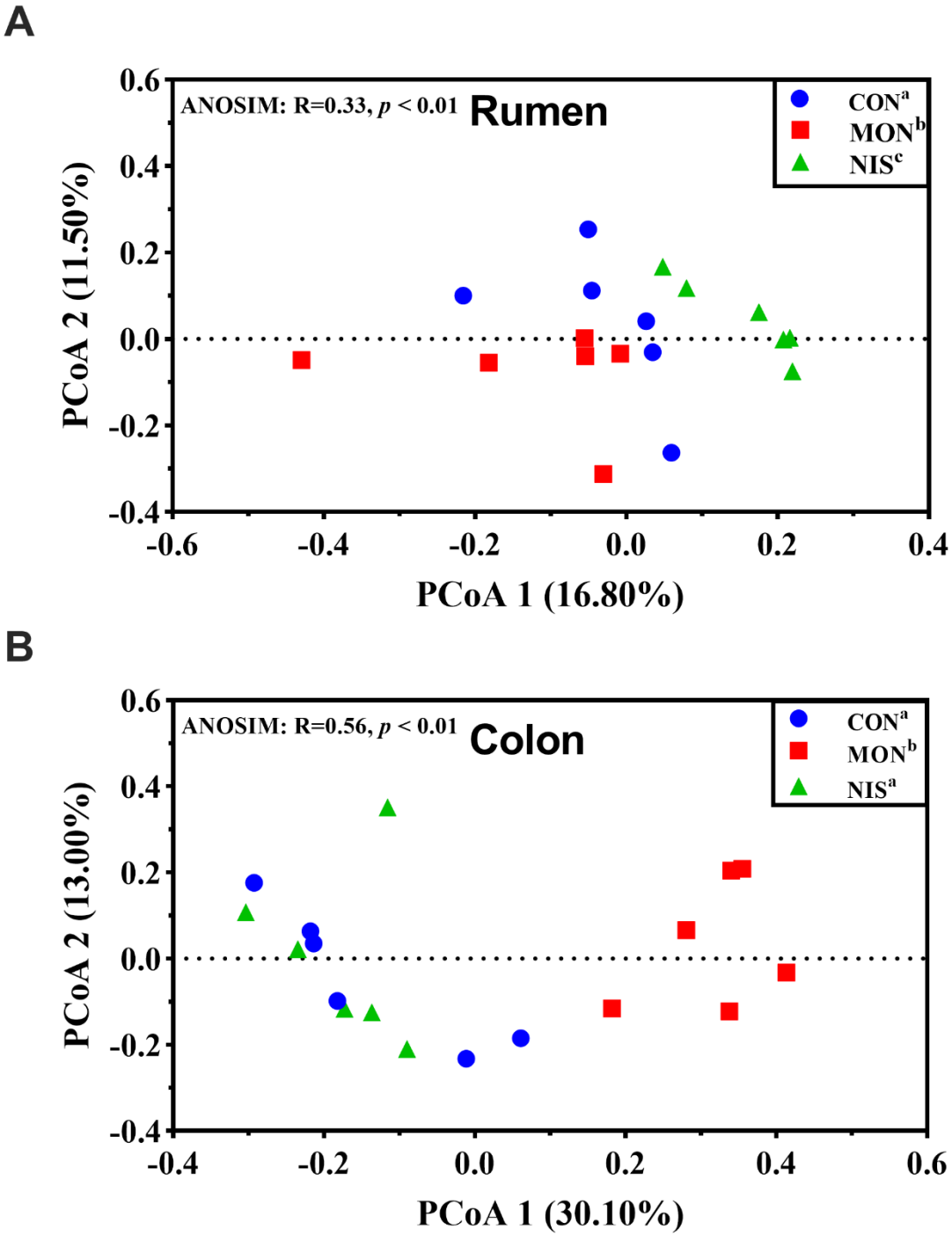
452

453 Figure 1. Effects of nisin and monensin on the absolute abundance of total bacteria, fungi, protozoa, and

454 methanogens in the rumen and colon of fattening Hu lambs, and the Y-axis represents the absolute abundance of

455 the target gene, expressed as log-transformed copy numbers per millilitre of sample. Bars with different letters

456 (a, b) indicate significant differences ($P < 0.05$).



457

458 Figure 2. PCoA analysis of rumen (A) and colon (B) bacterial community in fattening Hu lambs fed with

459 different supplements. Analysis of Similarities (ANOSIM) was used to evaluate the differences in bacterial

460 communities. Different shoulder scars indicate significant differences in bacterial communities ($p < 0.05$).

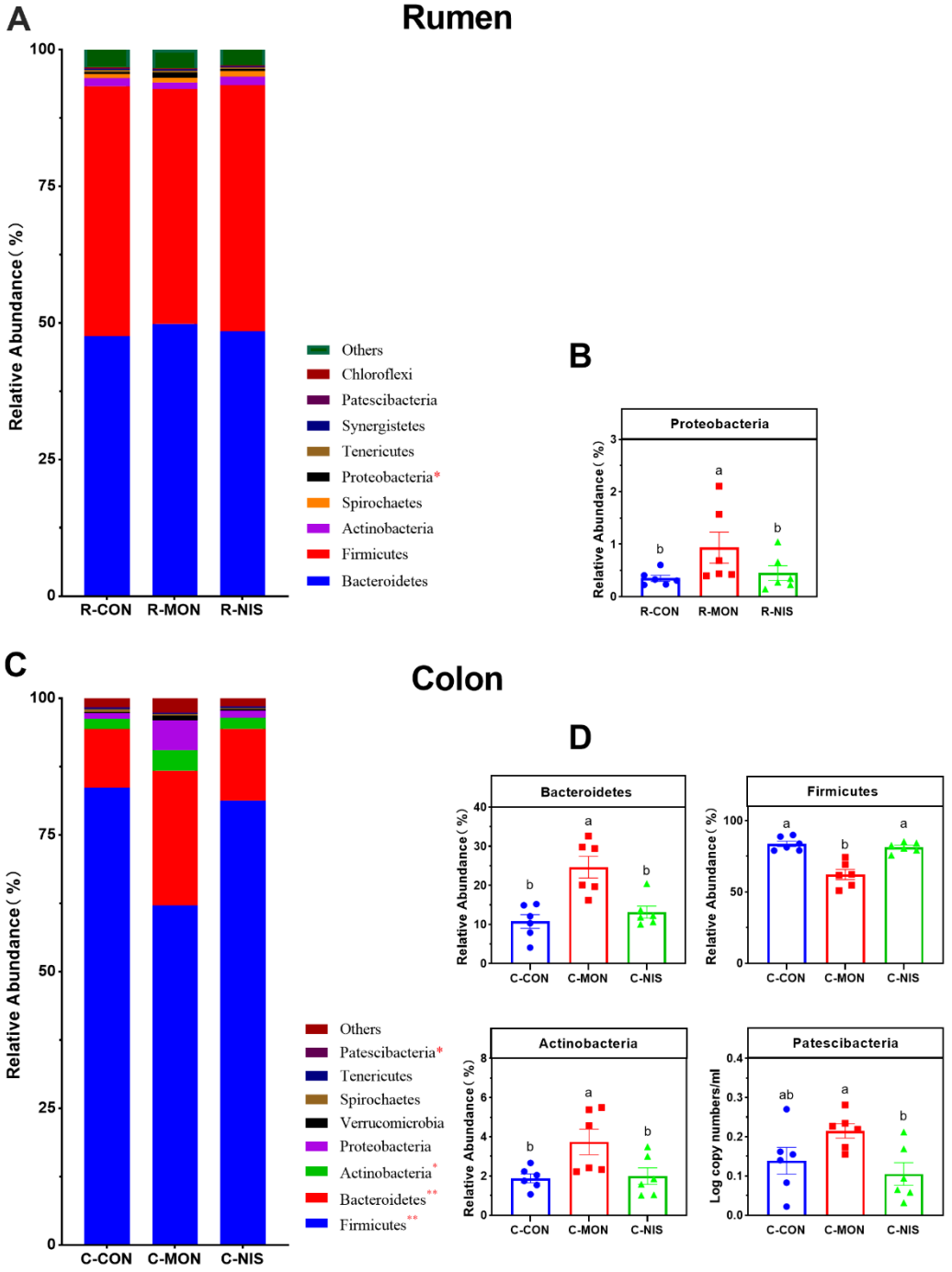


Figure 3. Effects of nisin and monensin on the relative abundance (%) of bacterial phyla (relative abundance >1% in at least one treatment) in rumen (A) and colon (C) of fattening Hu lambs. Representative bacterial phyla significantly affected in the rumen (B) and colon (D). Bacterial phyla in Fig. 3A and Fig. 3B with a * or ** deferred significantly ($p < 0.05$ and $p < 0.01$, respectively). Bards with different letters in Fig. 3B and D represent means that differ significantly ($p < 0.05$).

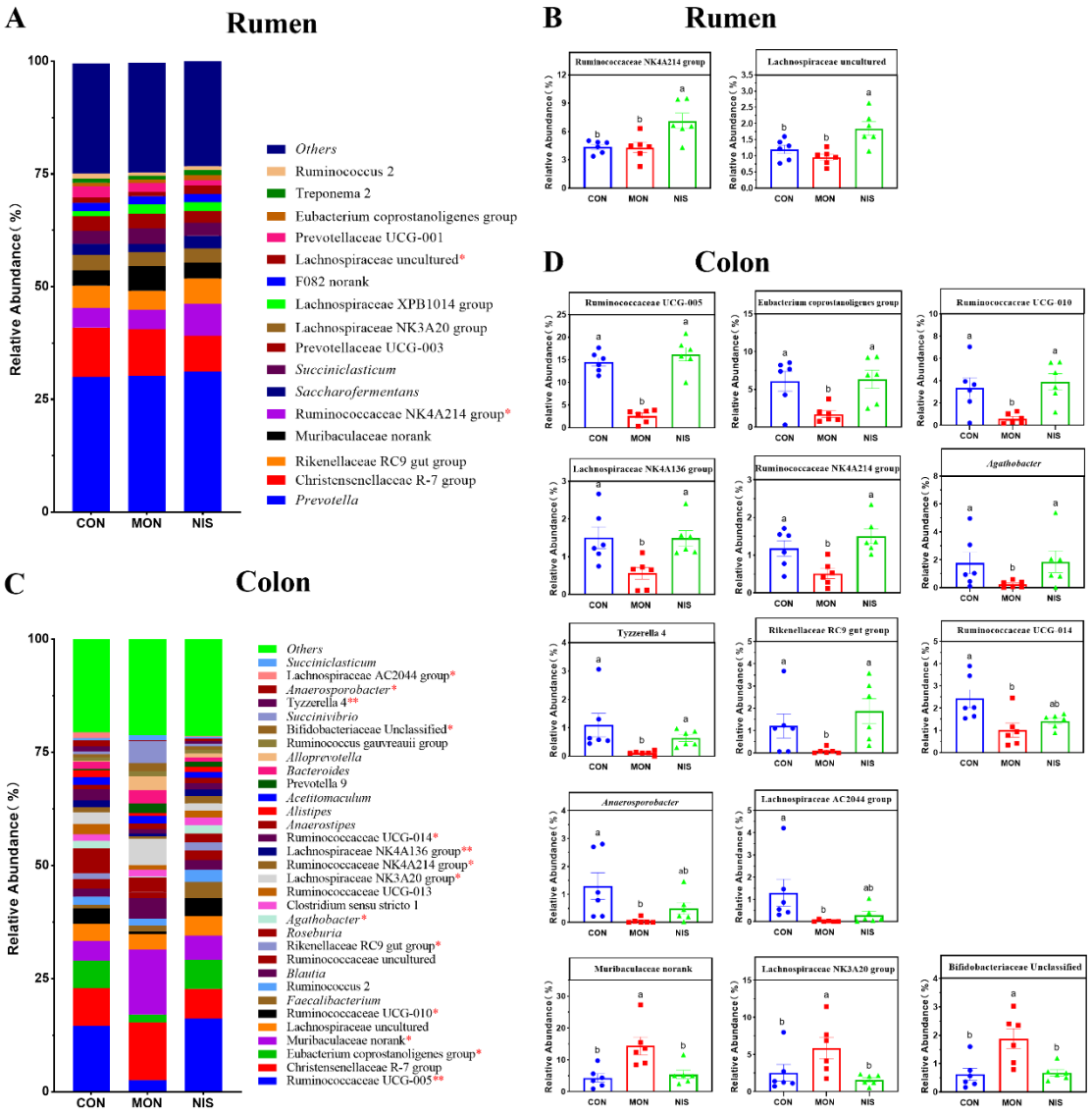


Figure 4. Effects of nisin and monensin on the relative abundance (%) of bacterial genera (only those with a relative abundance >1% in at least one treatment) in rumen (A) and colon (C) of fattening Hu lambs. Representative bacterial genera significantly affected in the rumen (B) and colon (D). Bacterial genera in Fig. 4A and Fig. 4C with a * or ** deferred significantly ($p < 0.05$ and $p < 0.01$, respectively). Bards with different letters in Fig. 4B and D represent means that differ significantly ($p < 0.05$).

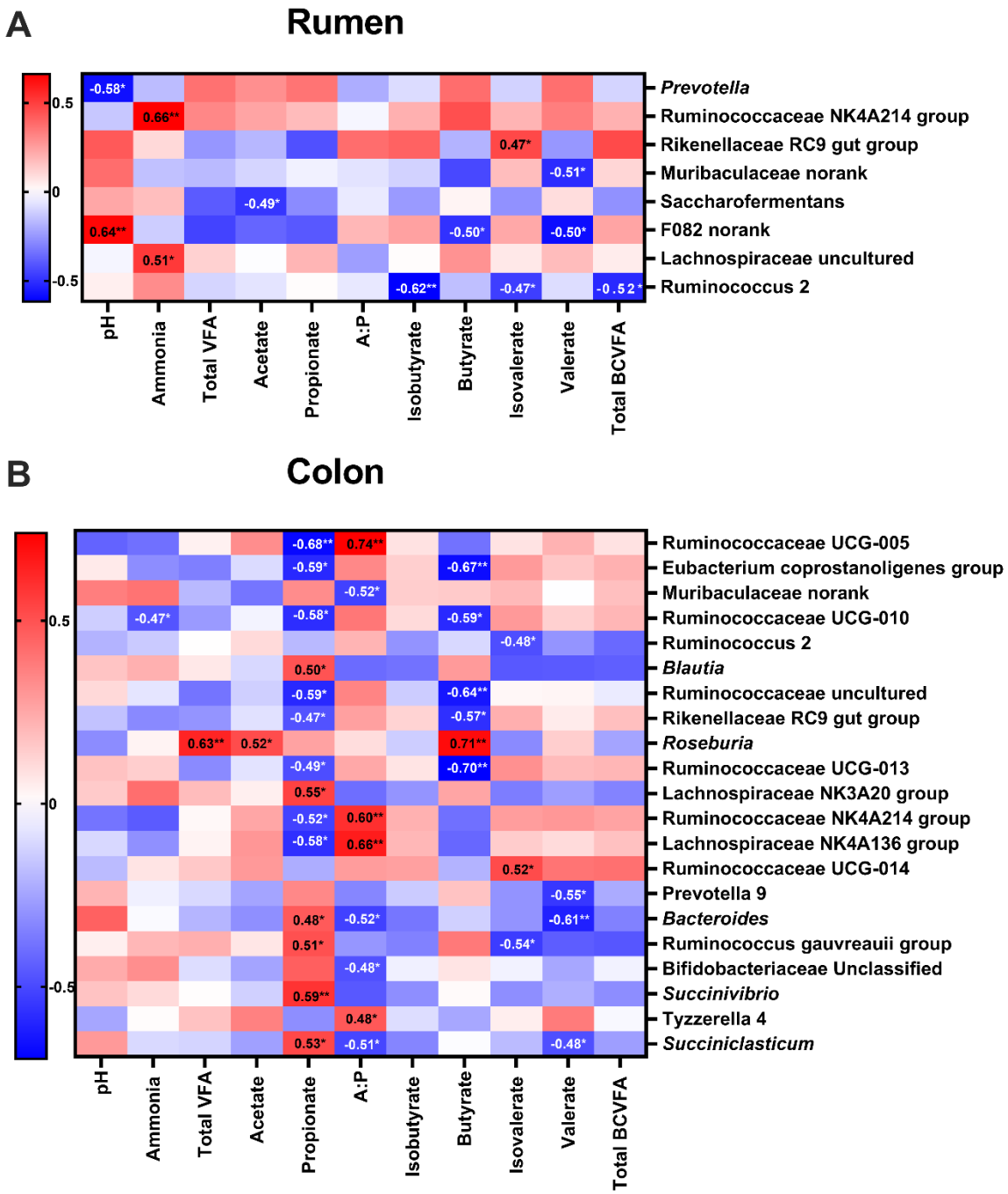


Figure 5. Correlations between the relative abundance of rumen (A) and colon (B) bacterial genera and fermentation parameters. *, significant correlation ($p < 0.05$). Only bacteria significantly associated with at least one of the fermentation characteristic indicators are shown.