

Impact of lysozyme on ruminal fermentation and microbial population in Rusitec fermenters

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ABSTRACT

The aim of the present study was to investigate the *in vitro* effects of two lysozyme doses (10 and 50 FIP U/mL) on rumen fermentation and microbial populations using the artificial rumen system Rusitec with an 80:20 concentrate-to-forage diet. The ruminal pH, the total number of protozoa, total and individual volatile fatty acid (VFA) production, and methane (CH₄) release were not significantly affected by the tested quantities of lysozyme. However, the ammonia nitrogen (NH₃-N) concentration was significantly increased by supplementation with Lysozyme-50 ($P < 0.05$). The dry matter digestibility (DMD) was significantly higher in Lysozyme-50 than in Lysozyme-10 ($P < 0.05$). The total bacterial count decreased linearly in response to the addition of Lysozyme-50 ($P < 0.05$). Furthermore, in the presence of Lysozyme-50, the abundance of *Butyrivibrio fibrisolvens* decreased in both linear and quadratic manners ($P < 0.05$), and the abundance of *Fibrobacter succinogenes* decreased in a linear manner ($P < 0.05$). A linear decline tendency in the cell numbers of *Ruminococcus albus* ($P = 0.092$) and *Megasphaera elsdenii* ($P = 0.085$) was observed with Lysozyme-50 treatment. These findings suggest that while core fermentation parameters such as VFA production and digestibility remained stable, the elevated ammonia nitrogen concentration may reflect reduced nitrogen utilization efficiency, particularly at high doses. Additionally, since lysozyme has limited selectivity for certain rumen bacteria within the Rusitec system, lysozyme may influence rumen microbes differently from conventional antibiotics, warranting further *in vitro* and *in vivo* studies across diverse diets and dose ranges.

Keywords: Enzymes, Feed additives, Rumen bacteria, Ruminal fermentation, Quantitative Real-Time PCR

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Received: 23.06.2025, Accepted: 23.11.2025, Publication Date: 01.04.2026

How to cite this article: Musa SAA, Demirtas A, Ozturk H (2026): Impact of lysozyme on ruminal fermentation and microbial population in Rusitec fermenters. Ankara Univ Vet Fak Derg, 73 (2), 145-153. DOI: 10.33988/auvfd.1725307.

Introduction

In the rumen, microbial fermentation of carbohydrates and proteins leads to losses of both energy and nitrogen. Approximately 2 to 12% of the energy derived from ingested feed is released as CH₄ by ruminants (16). In addition, a significant proportion of the nitrogen consumed by ruminants -between 75% and 85%- is excreted in faeces and urine (28). Energy and protein inefficiencies not only represent financial losses but also contribute to environmental pollution (23). Ruminants contribute to about 95% of the total enteric CH₄ emissions from animals worldwide, mainly due to their large population size, substantial body mass, and high feed intake (16). Consequently, various strategies are being

investigated to improve feed utilization and reduce the environmental impact of ruminant livestock production.

Antibiotics have been used for many years to enhance productivity in farm animals and have proven effective in improving the efficiency of livestock and poultry production. However, it is widely believed that using antibiotics as growth promoters leads to the emergence of antibiotic-resistant bacteria in farm animals (47). Consequently, the administration of antibiotics as supplements in animal production has been severely restricted or banned in several countries worldwide (47). Today, the priority is to use safe and natural alternatives, such as herbal extracts, essential oils, probiotics, prebiotics, and antimicrobial enzymes (23).

Lysozyme is an endogenous enzyme and a crucial component of innate immunity, primarily functioning as a hydrolytic enzyme that cleaves the β -(1,4)-glycosidic bond in peptidoglycan, thereby conferring antibacterial activity and causing cell lysis, particularly in Gram-positive bacteria (33). This selective activity against Gram-positive bacteria suggests that lysozyme could serve as an alternative antimicrobial feed additive, replacing ionophore antibiotics in livestock production. Due to its potent antimicrobial properties, the enzyme is frequently used as a natural preservative in the food industry (e.g., cheese, wine, and beer) and is widely applied in medicine and general veterinary and feed applications (20). Several studies have explored the potential impact of lysozyme on growth rates and changes in the microbiota of poultry (22, 52), pigs (30, 50, 54), fish (46, 53), and rabbits (18). Lysozyme is also used as an antibiotic alternative in veterinary applications, particularly for treating or preventing mastitis (2, 40, 43), and, in combination with other compounds, as an agent against bovine viral diarrhea virus (32). However, the impact of lysozyme on rumen fermentation and the microbial population has not been extensively studied. This gap is significant because compared with Gram-negative species, Gram-positive ruminal bacteria typically produce higher levels of ammonia, hydrogen, formate, and lactate (12). Accordingly, we hypothesised that the direct addition of lysozyme could alter the ruminal microbiome, thereby reducing methanogenesis and ammoniogenesis. Therefore, the aim of this study was to evaluate the *in vitro* effects of two doses of hen egg-white lysozyme on rumen fermentation and the rumen microbial population, and to demonstrate its potential as an alternative to banned antibiotics in livestock production.

Materials and Methods

Incubation Technique: The inoculum was prepared and the fermenters were established in Rusitec units (rumen simulation technique) according to the method described by Czerkawski and Breckenridge (11). Nine fermentation vessels were clamped into two Rusitec machines and immersed in a water bath maintained at 39°C and subjected to continuous vertical agitation. The effective volume of each vessel was 750 mL. A continuous flow of buffer solution was used to simulate rumen fluid turnover, maintain an environment similar to the *in vivo* rumen, and prevent loss of rumen bacteria. The buffer solution, prepared to a pH of 7.4 according to the method of McDougall (34) as modified by Öztürk (37), was delivered by a nine-channel electronic peristaltic pump (ISMATEC, IPC, Switzerland) to maintain a constant flow. Table 1 shows the composition of the buffer solution.

Table 1. The ingredients of the buffer solution (artificial saliva)

Ingredients	g/L
NaCl	1.636
KCl	0.57
1N HCl	0.5 mL
CaCl ₂ • 2H ₂ O	0.032
MgCl ₂ • 6H ₂ O	0.128
NH ₄ Cl	0.267
Na ₂ HPO ₄ • 12H ₂ O	3.581
NaH ₂ PO ₄ • H ₂ O	1.38
NaHCO ₃	8.225

Table 2. The composition of the barley straw hay and concentrate (as fed basis)

Ingredient	Concentrate	Barley straw hay
DM	91.12	94.19
Ash	6.50	5.82
CF	5.56	40.45
CP	12.42	2.95
EE	2.75	2.00
OM	84.62	88.37
NFC	61.44	16.43
NDF	16.89	72.80
ADF	7.41	49.98
ME (kcal/kg)	2655.25	1501.14

DM - Dry matter; CF - Crude fiber; CP - Crude protein; EE - Ether extract; OM - Organic matter; NFC - Non-fiber carbohydrate; NDF - Neutral-detergent fiber; ADF - Acid-detergent fiber; ME - Metabolic energy

An experiment using the Rusitec system was conducted with liquid (filtered and squeezed through gauze) and solid rumen contents collected from two adult cows (mean live body weight 450 kg) that had been freshly slaughtered at the Akyurt slaughterhouse in Ankara, Turkey. The inoculum was immediately delivered to the laboratory in a thermos container preheated to 39°C. The same ration administered to the animals was used in the *in vitro* experiment. Before being placed in food containers, the ration was weighed and packed into nylon bags (80 × 120 mm; pore size 150 μ m). The bags were then firmly secured with plastic ties. The composition of the barley straw hay and concentrate is shown in Table 2. On the first day of the study, each feed container received two nylon bags: one holding 80 g of wet rumen solids, and the other holding the daily diet. After 24 hours, the bags containing 80 g of wet rumen contents were replaced with fresh bags containing the daily diet, composed of concentrate and barley straw in an 80:20 ratio. A fresh nylon bag was placed in the container each day for the next 48 hours. The removed nylon bag was squeezed and rinsed twice with 20 mL of buffer solution, and the liquid fraction from the

wash was transferred back into the vessels. This procedure was necessary to avoid cumulative depletion of the microbial population as successively digested samples were removed (11). The washed nylon bag was placed in a polyethylene bag and frozen at -20°C for subsequent measurement of dry matter digestibility (DMD) percentage.

Experimental Procedure: The *in vitro* experiment was divided into two phases: a 6-day adaptation period aimed at establishing stable conditions, followed by a 6-day experimental period. A completely randomized design was employed, involving three distinct treatments. Each treatment was replicated three times to ensure statistical reliability and minimize experimental bias. The use of three treatment groups with three independent fermenters each ($n = 3$) is consistent with established Rusitec-based protocols (6, 8, 17, 21).

During the experimental period, each fermenter was supplemented daily with 1 mL, delivered directly into the vessel during feed-bag exchange. Three fermenters received 1 mL of distilled water without lysozyme (control group); three other fermenters received 1 mL of lysozyme solution at 10 FIP U/mL (Lysozyme-10); and the remaining three fermenters received 1 mL of lysozyme solution at 50 FIP U/mL (Lysozyme-50). The lysozyme used (LYSO-GUARD®), a hen egg-white preparation in liquid form containing approximately 9×10^6 FIP U/mL, was supplied by Mayasan Gıda San. ve Tic. A.Ş. (Istanbul, Turkey). Working solutions were prepared by diluting the stock solution with distilled water to achieve the target concentrations. Given the effective volume of each fermenter (750 mL), the final concentrations achieved in the fermenters were 10 FIP U/mL and 50 FIP U/mL, corresponding to daily additions of 7,500 and 37,500 FIP units per fermenter, respectively.

Sampling and Analytical Procedures: The ash, dry matter (DM), crude fiber (CF), crude protein (CP), and ether extract (EE) contents of the experimental diets (Table 2) were examined in accordance with the standards of the Association of Official Analytical Chemists (3). Measurements of organic matter (OM), non-fiber carbohydrates (NFC), neutral detergent fiber (NDF), acid detergent fiber (ADF), and metabolizable energy were performed as reported previously (13).

An Ion 6 pH meter (Acorn series, Oakton, VA, USA) was used to measure pH in each vessel daily when the nylon bags were changed. Every day, 1 mL of rumen fluid was sampled from each fermenter and carefully combined with 1 mL of a methyl green solution to count protozoa (13). The total number of protozoa was determined microscopically using a Fuchs-Rosenthal counting chamber (Leica CME, Morrisville, NC, USA). Two

samples were taken every day after the effluent volumes from each conical flask had been measured, and they were stored at -20°C for testing of ammonia nitrogen ($\text{NH}_3\text{-N}$) and volatile fatty acids (VFA). The conical flasks were flushed with CO_2 to maintain an optimal anaerobic environment for bacterial activity. Using the indophenol blue method (10), $\text{NH}_3\text{-N}$ concentrations were photometrically determined at 546 nm with a Shimadzu UV-150-02 double-beam UV spectrophotometer (Kyoto, Japan). According to Demirtas et al (13), gas chromatography (ACME-6100, Younglin, Republic of Korea) was used to measure the concentrations of volatile fatty acids. The daily production of VFA was determined by multiplying the corresponding VFA concentration by the amount of collected effluent. CH_4 production was calculated using the equations suggested by Abdl-Rahman (1), which are based on Wolin's stoichiometry (51).

The feed bags were dried at 65°C for 48 hours in an oven to determine the DMD. By subtracting the weight of the dry matter residue from the weight of the original dry matter sample, dividing the resulting difference by the weight of the original dry matter sample, and multiplying the result by 100, the percentage of DMD after 48 hours was determined (37).

DNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Assay: On the sixth day of the experiment, samples were taken from the Rusitec fermenters for DNA extraction to characterise the rumen bacteria. These samples were taken while the feed bags were being exchanged, to ensure that they included both planktonic and solid-phase bacteria (49). Immediately following collection, samples were placed in liquid nitrogen. Before being processed further, the samples were stored at -20°C . After thawing, the samples were centrifuged at 10,000 rpm at room temperature for 10 minutes. The pellets were then redissolved in saline buffered with $2 \times$ phosphate (26). An E.Z.N.A.™ stool DNA extraction kit (Omega Bio-Tek, USA) was employed to isolate total DNA from the pellets. The Take3 plate of a microplate reader (Epoch, Bio-Tek, USA) was used to spectrophotometrically assess the DNA concentration and purity at A260/280.

A quantitative real-time PCR experiment using specific primers was conducted to ascertain the population dynamics and abundance of typical rumen bacteria (Table 3). As previously described (12), reactions were carried out using the SYBRGreen I master mix (Roche, 04707516001) on a LightCycler 480II Real-Time PCR system (Roche). Melting curve analysis confirmed the presence of a single amplification product. Calibration curves obtained from the Cp values of microbial DNA, generated from 10^2 to 10^{10} cells/mL of a reference microbe for each target species, were used to calculate microbial quantities (12).

Table 3. Primers used for Real-Time PCR (qRT-PCR) assay

Target species	Forward primer (5'-3')	Reverse primer (5'-3')	References
Total bacteria (16S rRNA)	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	(14)
Methanogenic archaea (<i>mcrA</i>)	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	(15)
HAP bacteria (16S rDNA)	GAGTTTGATCCTGGCTCAG	AAGGAGGTGATCCAGCC	(5)
<i>R. albus</i> (16S rDNA)	CAAAACCCTAAAAGCAGTCTTAGTTCG	GACGGGCGGTGTGTACAAG	(29)
<i>R. flavefaciens</i> (16S rRNA)	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC	(14)
<i>B. fibrisolvens</i> (16S rDNA)	ACACACCGCCCGTCACA	TCCTTACGGTTGGGTCACAGA	(27)
<i>F. succinogenes</i> (16S rRNA)	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCCTGAACTATC	(14)
<i>M. elsdenii</i> (16S rDNA)	GACCGAAACTGCGATGCTAGA	CGCCTCAGCGTCAGTTGTC	(36)
<i>S. ruminantium</i> (16S rDNA)	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	(44)

HAP bacteria - Hyper-ammonia producing bacteria

Statistical Analysis: A two-way repeated-measures analysis of variance (ANOVA) was used to examine the data from Rusitec. Fermenters were regarded as random effects; treatment, day, and their interaction were regarded as fixed effects; and sampling day was regarded as a repeated measure. For statistical analysis, the individual fermenter served as the experimental unit. Duncan's multiple range test was employed to detect differences among treatment means. The analyses were conducted using the statistical program SigmaStat 3.1 (Systat Software, Erkrath, Germany). When $P < 0.05$, differences were classified as statistically significant. Data on rumen microorganisms were log₁₀-transformed before statistical analysis. The linear and quadratic effects of lysozyme dose on rumen microbial populations in the Rusitec were evaluated using polynomial regression with sequential analysis of variance. The significance of differences among doses was assessed using a general linear model (GLM). When substantial differences were evident, Fisher's least significant difference (LSD) test was employed to differentiate between means. For all analyses, a tendency was identified when $0.05 < P < 0.10$, and differences were considered statistically significant at $P \leq 0.05$.

Results

As shown in Table 4, the addition of lysozyme did not result in significant changes in ruminal pH, total and individual VFA production, CH₄ production, or total

protozoa counts. Although DMD did not differ significantly between the control group and the Lysozyme groups, DMD was higher in Lysozyme-50 than in Lysozyme-10. Additionally, Lysozyme-50 led to a significant increase in the NH₃-N concentration ($P < 0.05$) compared with the control group. Furthermore, sampling days had a statistically significant impact on the fermentation parameters ($P < 0.05$), with the exception of ruminal pH and DMD.

Lysozyme supplementation altered the abundance of certain microbial populations in Rusitec fermenters, as illustrated in Figure 1. The total bacteria count exhibited a linear decrease in response to the addition of Lysozyme-50 ($P < 0.05$). Furthermore, this addition led to linear and quadratic decreases in the cell numbers of *Butyrivibrio fibrisolvens* ($P < 0.05$), and to a non-significant trend toward a linear decrease in the abundance of the Gram-positive bacterium *Ruminococcus albus* ($P = 0.092$). Treatment with Lysozyme-50 had similar effects on Gram-negative rumen bacteria; *Fibrobacter succinogenes* was significantly less abundant in the Lysozyme-50 group ($P < 0.05$). Additionally, *F. succinogenes* cell counts were statistically comparable between the Lysozyme-10 and the Lysozyme-50 treatments. A non-significant trend toward a linear decrease in *Megasphaera elsdenii* cell counts was also observed with Lysozyme-50 treatment ($P = 0.085$). However, the quantities of methanogenic archaea, HAP bacteria, *S. ruminantium*, and *R. flavefaciens* were not substantially affected by lysozyme supplementation.

Table 4. Effects of lysozyme on rumen fermentation characteristics in Rusitec fermenters

Item	Control	Lysozyme-10	Lysozyme-50	SEM	P		
					T	D	T × D
Ruminal pH	6.56	6.55	6.54	0.01	0.483	0.507	0.995
Total VFA (mmol/d)	32.88	32.91	34.01	1.14	0.740	<0.001	0.519
Acetate	17.30	17.24	17.63	0.55	0.865	<0.001	0.301
Propionate	9.34	9.37	9.76	0.43	0.832	<0.001	0.911
Butyrate	6.25	6.30	6.62	0.29	0.747	<0.001	0.707
CH ₄ (mmol/d)	9.44	9.43	9.69	0.30	0.863	<0.001	0.189
NH ₃ -N (mmol/L)	9.52 ^b	9.66 ^b	10.17 ^a	0.16	0.014	0.002	0.966
Protozoa (×10 ³ /mL)	18.99	18.13	17.12	2.30	0.961	0.041	0.959
DMD (%)	70.50 ^{ab}	68.98 ^b	72.16 ^a	1.42	0.021	0.112	0.950

a,b – Means in the same row with different superscripts are significantly different ($P < 0.05$). Lysozyme-10 – 10 FIP U/mL lysozyme; Lysozyme-50 – 50 FIP U/mL lysozyme; VFA - Volatile fatty acids; DMD - Dry matter digestibility; SEM - Standard error of the mean; T - treatment; D - day; T × D - treatment × day interaction

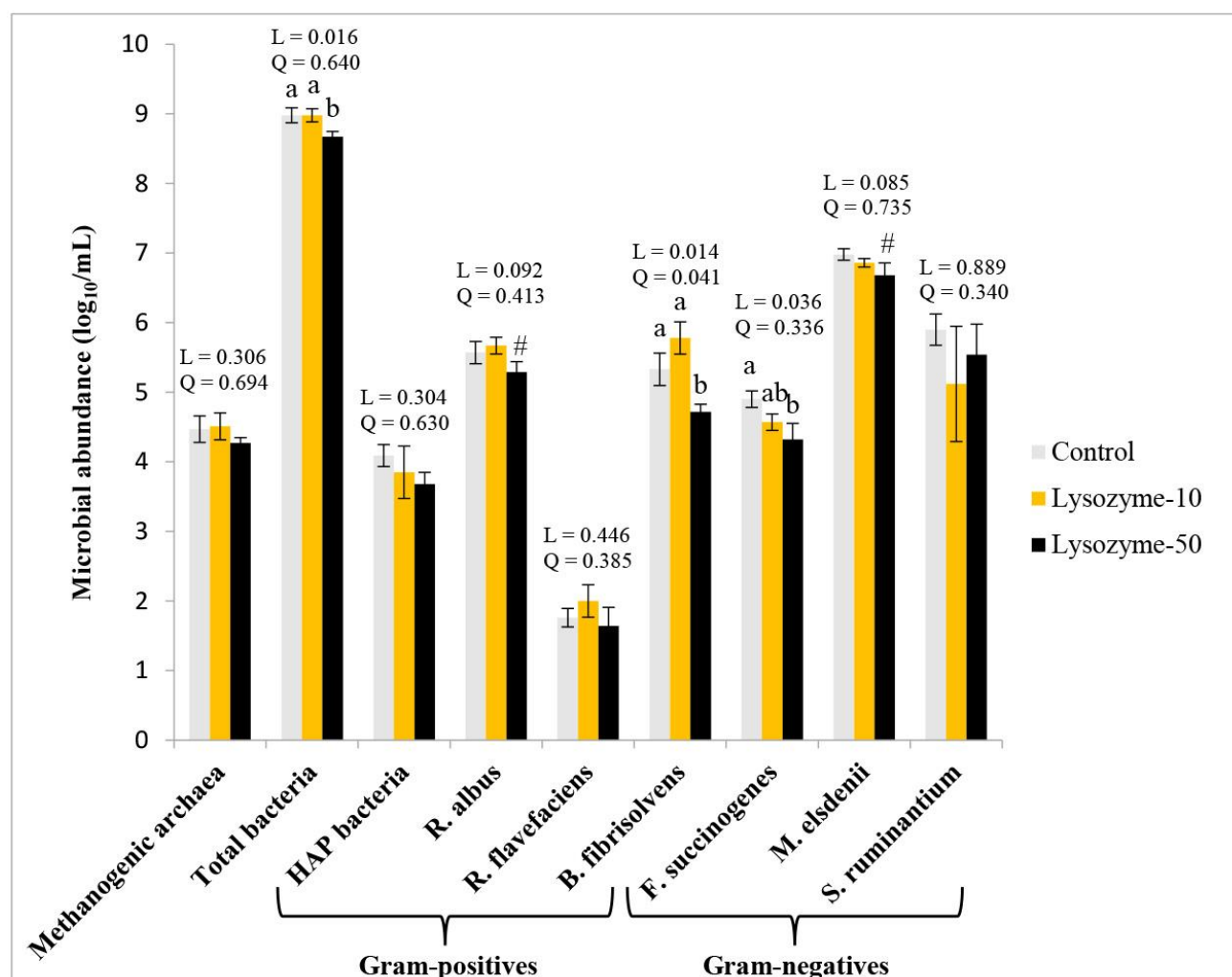


Figure 1. Effects of lysozyme on the abundance of representative rumen microorganisms in Rusitec fermenters determined by real-time PCR. Graph shows means and standard error of the means. ab – Bars with different superscripts for each microorganism differ at $P \leq 0.05$; # - Indicates a tendency toward significance ($0.05 < P < 0.10$); L - Probability of a linear effect; Q - Probability of a quadratic effect; Lysozyme-10 – 10 FIP U/mL lysozyme; Lysozyme-50 – 50 FIP U/mL lysozyme; HAP bacteria - Hyper-ammonia producing bacteria.

Discussion and Conclusion

Lysozyme, an antimicrobial proteolytic enzyme, is found in the diverse range of biological fluids and tissues, including plants, bird eggs, polymorphonuclear leukocyte granules, and animal secretions such as milk, saliva, tears, and respiratory secretions (25). Lysozyme is also called muramidase because its natural substrate is peptidoglycan, also known as murein, the principal component of the bacterial cell wall. Thus, lysozyme-induced peptidoglycan hydrolysis causes bacterial mortality and cell lysis (33). Lysozyme has been investigated as a potential substitute for antibiotic growth promoters to enhance animal performance because of its antibacterial properties and low toxicity (4). In this study, we examined the impact of supplementing diets with two levels of lysozyme (Lysozyme-10 and Lysozyme-50) on microbial populations and ruminal fermentation.

In the current study, a high-concentrate diet (80:20 concentrate-to-forage ratio) was utilized to represent a specific feeding system prevalent in certain production settings. Since microbial composition and fermentation dynamics vary significantly between forage-rich and high-concentrate diets, the findings should be considered within the context of this high-concentrate diet, and it must be acknowledged that they may not be generalizable across all ruminant feeding systems. This limitation has been taken into account during the interpretation and discussion of the results.

The two doses (10 and 50 FIP U/mL) were selected based on a preliminary assessment of concentrations used in similar *in vitro* fermentation studies, and were intended to represent a low and a high dose within a practically testable range (7). The Lysozyme-50 dose was specifically chosen to assess the effect of a substantially elevated lysozyme concentration on a broader range of ruminal bacteria, given lysozyme's preferential activity against Gram-positive bacteria.

The microbial communities in the fermenters were slightly affected by a particularly high dose of lysozyme supplementation in this study. The total bacterial count was lower with Lysozyme-50 supplementation. Biswas et al (7) also observed a tendency for decreased bacterial DNA copy numbers in the lysozyme-treated groups (2,000, 4,000, and 8,000 U) during a short-term *in vitro* rumen fermentation assay. In this study, Lysozyme-50 specifically reduced the populations of the Gram-positive bacterium *B. fibrisolvens* and the Gram-negative bacterium *F. succinogenes*. Non-significant trends toward decreases were also noted for *R. albus* and *M. elsdenii*. The observed decline in the abundance of significantly affected Gram-positive and Gram-negative species suggests that lysozyme may have limited selectivity when acting on bacterial populations in Rusitec fermenters. Gram-positive bacteria are generally reported to be more

susceptible to lysozyme than Gram-negative bacteria, owing to their thicker peptidoglycan layer containing teichoic acids in their cell walls (33). Another reason is that Gram-negative bacteria, unlike Gram-positive bacteria, possess an outer membrane composed mainly of lipopolysaccharides (LPS). This membrane prevents lysozyme from accessing and lysing the peptidoglycan of the cell wall (24). However, several studies have shown that lysozyme's bactericidal effectiveness stems from both its lytic activity and its cationic and hydrophobic characteristics (38). Cationic regions of lysozyme are able to bind the anionic core components of LPS molecules and increase the permeability of the outer membrane of Gram-negative bacteria to hydrophobic compounds (19). In addition to this hypothesis, it should be noted that lysozyme, a polypeptide, may degrade under rumen conditions, potentially limiting its effectiveness against Gram-positive bacteria. A clear understanding of lysozyme's mechanism of action requires more comprehensive sequencing of rumen bacteria.

The levels of total and individual volatile fatty acids were not affected by lysozyme supplementation at any dose in this study. This observation suggests that ruminal fermentation was not suppressed despite reductions in the abundance of total bacteria and of some bacterial species induced by Lysozyme-50. The extant literature on the effects of lysozyme on rumen fermentation and bacterial populations is limited. However, Biswas et al. (7) reported higher levels of total VFA, acetic acid, and propionic acid after 24 hours of incubation. Lysozyme supplementation also decreased CH₄ production in that study (7), whereas CH₄ production remained unchanged in the present study. The observed variations in outcomes might be attributable to discrepancies in incubation durations across the two studies. On the other hand, the absence of an effect on CH₄ production was consistent with the unchanged abundance of methanogenic archaea observed in the present study.

In this study, the DMD remained unchanged in the groups treated with lysozyme relative to the control group. This finding is in line with Biswas et al. (7), who also found no effect of lysozyme on DMD. However, DMD was higher with Lysozyme-50 than with Lysozyme-10 in the present study. It has also been reported that lysozyme participates in digestion by exhibiting enzymatic activity in certain animal species (41). Nevertheless, the data from this study are not sufficient to support such a conclusion.

In the present study, lysozyme supplementation did not affect ruminal pH. The ideal pH for the proliferation of cellulolytic bacteria in the rumen ranges from 6.5 to 6.8 (42). The pH values of the control, Lysozyme-10, and Lysozyme-50 groups were 6.56, 6.55, and 6.54, respectively; all within the physiological range. This result is likely attributable to the absence of large quantities of acid end products during ruminal fermentation, primarily

because the Rusitec system is more buffered than the *in vivo* rumen. In contrast, Biswas et al (7) reported that after 24 hours of incubation, 8,000 U of lysozyme increased the pH. However, the observed increase was suggested to result from the high pH (8.99) of the lysozyme used.

Ammonia plays a pivotal role in ruminal nitrogen metabolism as an intermediary; its concentration is influenced by the rate of ammonia production, primarily from amino acid fermentation and urea hydrolysis, and by the rate at which it is incorporated into microbial protein (9). Hence, an increased ammonia concentration indicates lower microbial protein synthesis efficiency, whereas a decreased ammonia concentration indicates improved nitrogen utilization efficiency (12). According to previous reports, the optimal ruminal $\text{NH}_3\text{-N}$ concentration for maintaining proper fermentation and nutrient utilization is considered to be within 8.5-30 mg/dL (45). In the present study, the $\text{NH}_3\text{-N}$ concentrations obtained in all experimental groups were also within this optimal range. Fermenters that received Lysozyme-50 showed a significant elevation in $\text{NH}_3\text{-N}$ concentration and a decrease in total bacterial abundance. This result is consistent with the findings of Biswas et al (7), who observed that 8,000 U of lysozyme caused an elevation in $\text{NH}_3\text{-N}$ concentration and a decrease in bacterial DNA copy numbers. The elevated $\text{NH}_3\text{-N}$ concentration is attributable to reduced ammonia utilization by rumen microorganisms, as evidenced by a decrease in total bacteria in the present study. In addition, the elevated $\text{NH}_3\text{-N}$ concentration can be attributed to increased protein hydrolysis. This is noteworthy because lysozyme, a polypeptide composed of 129 amino acids (48), is susceptible to degradation under rumen conditions owing to the high hydrolytic activity of rumen microorganisms.

Protozoa play a significant role in the rumen ecosystem, where they prey on bacteria and influence nutrient flow and methane production (31, 35). In the present study, lysozyme supplementation did not significantly affect the total number of protozoa, which is consistent with the lack of effect observed on CH_4 production. This outcome is biologically plausible, as lysozyme primarily targets the peptidoglycan layer of bacterial cell walls, whereas rumen protozoa are eukaryotic organisms lacking this structure (39). Biswas et al (7) also reported that 2,000, 4,000, or 8,000 units of lysozyme had no significant effect on protozoan DNA copy number *in vitro*.

In conclusion, lysozyme supplementation had measurable but limited effects in the Rusitec system. Although total VFA production was maintained, the high dose (Lysozyme-50) resulted in a significant decrease in total bacteria, including certain Gram-positive and Gram-negative species, and a significant increase in $\text{NH}_3\text{-N}$ concentration, suggesting a potential reduction in nitrogen

utilization efficiency. The limited specificity of lysozyme against selected Gram-positive bacteria within the Rusitec system, particularly at the high dose, suggests that it may affect rumen bacteria differently from conventional antibiotics. It is important to note that these findings are limited to the Rusitec *in vitro* system and to the high-concentrate (80:20) diet tested. Another limitation of the study is that only the effects of selected low- and high-dose levels were examined. To validate lysozyme's potential as a natural feed additive in ruminants, further *in vitro* and *in vivo* studies are required, particularly those exploring broader dose ranges and diverse dietary regimens, and employing more comprehensive sequencing of rumen bacteria to assess its stability and dose-response dynamics.

Acknowledgements

This work is based on the Master's thesis of Saad Ahmed Adam MUSA, submitted to the Graduate School of Health Sciences, Ankara University, titled "Effects of Lysozyme on Rumen Microbial Fermentation Using Rumen Simulation Technique (RUSITEC)".

Financial Support

This study was conducted without financial support from any public or private funding bodies.

Ethical Statement

There are no ethical considerations relevant to the content or execution of this study.

Conflict of Interest

No conflicts of interest are reported by the authors with regard to this manuscript.

Author Contributions

SAAM was involved in all stages of the research. AD contributed to the PCR analyses. HO served as the supervisor and provided guidance during the writing and revision of the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

References

1. **Abdl-Rahman MA** (2010): *In vitro* manipulation of rumen fermentation efficiency by fumaric acid-bentonite coupled addition as an alternative to antibiotics. J Agric Sci, 2, 174-180.
2. **Adduci F, Elshafie HS, Labella C, et al** (2019): Abatement of the clostridial load in the teats of lactating cows with lysozyme derived from donkey milk. J Dairy Sci, 102, 6750-6755.

3. AOAC (Association of Official Analytical Chemists) (2000): Official Methods of Analysis of AOAC International. 17th ed. AOAC, USA.
4. Aratboni HA, Olvera C, Ayala M (2024): Nanoformulations for lysozyme-based additives in animal feed: An alternative to fight antibiotic resistance spread. *Nanotechnol Rev*, **13**, 20240015.
5. Attwood GT, Klieve AV, Ouwerkerk D, et al (1998): Ammonia-hyperproducing bacteria from New Zealand ruminants. *Appl Environ Microb*, **64**, 1796-804.
6. Benhissi H, de Heredia, IB, García-Rodríguez A (2016): Effect of replacing palm fat with high-linoleic cold-pressed rapeseed or sunflower cakes on fatty acid biohydrogenation in an artificial rumen (Rusitec). *Anim Prod Sci*, **58**, 499-506.
7. Biswas AA, Lee SS, Mamuad LL, et al (2016): Use of lysozyme as a feed additive on in vitro rumen fermentation and methane emission. *Asian-Australas J Anim Sci*, **29**, 1601-1607.
8. Boguhn J, Kluth H, Rodehutsord M (2006): Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. *J Dairy Sci*, **89**, 1580-1591.
9. Busquet M, Calsamiglia S, Ferret A, et al (2005): Effect of garlic oil and four of its compounds on rumen microbial fermentation. *J Dairy Sci*, **88**, 4393-4404.
10. Chaney AL, Marbach EP (1962): Modified reagents for determination of urea and ammonia. *Clin Chem*, **8**, 130-132.
11. Czerkawski JW, Breckenridge, G (1977): Design and development of a long-term rumen simulation technique (RUSITEC). *Br J Nutr*, **38**, 371-384.
12. Demirtas A, Ozturk H, Sudagidan M, et al (2019): Effects of commercial aldehydes from green leaf volatiles (green odour) on rumen microbial population and fermentation profile in an artificial rumen (Rusitec). *Anaerobe*, **55**, 83-92.
13. Demirtas A, Sevin S, Musa SAA, et al (2023): Preliminary study of the effects of *Apilactobacillus kunkeei* EIR/BG-1 and *Enterococcus hirae* EIR/CM-2 supplementation on in vitro rumen fermentation and microbial population. *J Anim Feed Sci*, **33**, 88-100.
14. Denman SE, McSweeney CS (2006): Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol*, **58**, 572-582.
15. Denman SE, Tomkins NW, McSweeney CS (2007): Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol*, **62**, 313-322.
16. Donald EJ, Ward GM (1996): Estimates of animal methane emissions. *Environ Monit Assess*, **42**, 133-141.
17. Duarte AC, Holman DB, Alexander TW, et al (2017): Incubation temperature, but not pequi oil supplementation, affects methane production, and the ruminal microbiota in a rumen simulation technique (Rusitec) system. *Front Microbiol*, **8**, 1076.
18. El-Deep MH, Amber KA, Eid YZ, et al (2021): The influence of chicken egg lysozyme or zinc-bacitracin antibiotic on the growth performance, antibacterial capacity, blood profiles, and antioxidative status of rabbits: A comparative study. *Animals*, **11**, 1731.
19. Ellison R, Giehl TJ (1991): Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest*, **88**, 1080-1091.
20. Ferraboschi P, Ciceri S, Grisenti P (2021): Applications of lysozyme, an innate immune defense factor, as an alternative antibiotic. *Antibiotics*, **10**, 1534.
21. Goiri I, Garcia-Rodriguez A, Oregui LM (2009): Effect of chitosan on mixed ruminal microorganism fermentation using the rumen simulation technique (Rusitec). *Anim Feed Sci Tech*, **152**, 92-102.
22. Gong M, Anderson D, Rathgeber B, et al (2017): The effect of dietary lysozyme with EDTA on growth performance and intestinal microbiota of broiler chickens in each period of the growth cycle. *J Appl Poult Res*, **26**, 1-8.
23. Hristov AN, Firkins JL, Dijkstra J, et al (2013): Special topics-mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *J Anim Sci*, **91**, 5045-5069.
24. Ibrahim HR, Kato A, Kobayashi K (1991): Antimicrobial effects of lysozyme against gram-negative bacteria due to covalent binding of palmitic acid. *J Agric Food Chem*, **39**, 2077-2082.
25. Ibrahim HR, Matsuzaki T, Aoki T (2001): Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *FEBS Letters*, **506**, 27-32.
26. Khafipour E, Li S, Plaizier JC, et al (2009): Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl Environ Microbiol*, **75**, 7115-7124.
27. Klieve AV, Hennessy D, Ouwerkerk D, et al (2003): Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. *J Appl Microbiol*, **95**, 621-630.
28. Kohn R, Dinneen M, Russek-Cohen E (2005): Using blood urea nitrogen to predict nitrogen excretion and efficiency of nitrogen utilization in cattle, sheep, goats, horses, pigs, and rats. *J Anim Sci*, **83**, 879-889.
29. Li X, Durmic Z, Liu S, et al (2014): *Eremophila glabra* reduces methane production and methanogen populations when fermented in a Rusitec. *Anaerobe*, **29**, 100-107.
30. Long Y, Lin S, Zhu J, et al (2016): Effects of dietary lysozyme levels on growth performance, intestinal morphology, non-specific immunity and mRNA expression in weanling piglets. *Anim Sci J*, **87**, 411-418.
31. López-García A, Saborío-Montero A, Gutiérrez-Rivas M, et al (2022): Fungal and ciliate protozoa are the main rumen microbes associated with methane emissions in dairy cattle. *GigaScience*, **11**, giab088.
32. Malaczewska J, Kaczorek-Łukowska E, Wójcik R, et al (2019): Antiviral effects of nisin, lysozyme, lactoferrin and their mixtures against bovine viral diarrhoea virus. *BMC Vet Res*, **15**, 1-12.
33. Masschalck B, Michiels CW (2003): Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Crit Rev Microbiol*, **29**, 191-214.
34. McDougall EI (1948): Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem J*, **43**, 99-109.

35. Newbold CJ, Ramos-Morales E, O'Hara E (2015): Rumen protozoa and methanogenesis: Not a simple cause-effect relationship. *Brit J Nutr*, **114**, 374-377.
36. Ouwerkerk D, Klieve AV, Forster RJ (2002): Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. *J Appl Microbiol*, **92**, 753-758.
37. Öztürk H (2003): *In vitro* studien zum einfluss von topinamburmehl und *Saccharomyces boulardii* auf den mikrobiellen vorgagenstoffwechsel. Dissertation, Tierärztliche Hochschule Hannover, Germany.
38. Pellegrini A, Thomas U, Von Fellenberg R, et al (1992): Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. *J Appl Microbiol*, **72**, 180-187.
39. Ragland SA, Criss AK (2017): From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathog*, **13**, e1006512.
40. Rathi CL, Risbud SP, Manohar BM (2008): Novel compositions comprising lysozyme and serratiopeptidase for prevention and treatment of mastitis and metritis. Patent No. WO2008035370. Available at <https://patentscope.wipo.int/search/en/detail.jsf?docId=W02008035370&form=MG0AV3>. (Accessed July 14, 2025).
41. Sahoo NR, Kumar P, Bhusan B, et al (2012): Lysozyme in livestock: A guide to selection for disease resistance: A review. *J Anim Prod. Adv*, **2**, 347-360.
42. Shi Y, Weimer P (1992): Response surface analysis of the effects of pH and dilution rate on *Ruminococcus flavefaciens* FD-1 in cellulose-fed continuous culture. *Appl Environ Microbiol*, **58**, 2583-2591.
43. Smulski S, Gehrke M, Libera K, et al (2020): Effects of various mastitis treatments on the reproductive performance of cows. *BMC Vet Res*, **16**, 1-10.
44. Tajima K, Aminov RI, Nagamine T, et al (2001): Diet dependent shifts in the bacterial population of the rumen revealed with Real- Time PCR. *Appl Environ Microbiol*, **67**, 2766-2774.
45. Uğurlu M, Uysal A, Nacar B, et al (2025): Effect of breed and fattening system on fattening performance, rumen and blood parameters in Akkaraman, Karayaka and Herik lambs under indoor conditions. *Ankara Univ Vet Fak Derg*, **72**, 397-404.
46. Verlhac V (2017): Feed additives for aquatic animals comprising essential oils and lysozyme. Patent No. WO2017064092. Available at <https://patentscope.wipo.int/search/en/detail.jsf?docId=W02017064092&form=MG0AV3>. (Accessed July 14, 2025).
47. Vieco-Saiz N, Belguesmia Y, Raspoet R, et al (2019): Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Front Microbiol*, **10**, 57.
48. Vilcacundo R, Méndez P, Reyes W, et al (2018): Antibacterial activity of hen egg white lysozyme denatured by thermal and chemical treatments. *Sci Pharm*, **86**, 48.
49. Watanabe Y, Suzuki R, Koike S, et al (2010): *In vitro* evaluation of cashew nut shell liquid as a methane-inhibiting and propionate-enhancing agent for ruminants. *J Dairy Sci*, **93**, 5258-5267.
50. Wells JE, Berry ED, Kalchayanand N, et al (2015): Effect of lysozyme or antibiotics on faecal zoonotic pathogens in nursery pigs. *J Appl Microbiol*, **118**, 1489-1497.
51. Wolin MJ (1960): A theoretical rumen fermentation balance. *J Dairy Sci*, **43**, 1452-1459.
52. Xia Y, Kong J, Zhang G, et al (2019): Effects of dietary supplementation with lysozyme on the structure and function of the cecal microbiota in broiler chickens. *PLoS One*, **14**, e0216748.
53. Yousefi M, Vatnikov YA, Kulikov EV, et al (2020): The protective effects of dietary garlic on common carp (*Cyprinus carpio*) exposed to ambient ammonia toxicity. *Aquaculture*, **526**, 735400.
54. Zou L, Xiong X, Liu H, et al (2019): Effects of dietary lysozyme levels on growth performance, intestinal morphology, immunity response and microbiota community of growing pigs. *J Sci Food Agric*, **99**, 1643-1650.

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