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Multi-strain Synbiotic Supplementation Improves Rumen Fermentation and Production Performance in Early Lactation Dairy Cows

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Abstract

This study evaluated the effects of graded levels of a multi-strain synbiotic on rumen fermentation, microbial populations, and production performance in early-lactation Holstein cows. Thirty-two cows were assigned to a randomized complete block design with four treatments: control (basal TMR) or synbiotic at 5, 10, or 15 g/cow/day from parturition to 100 days in milk. The synbiotic contained *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Bacillus subtilis*, mannan-oligosaccharides, and β-glucans. Rumen fermentation parameters were measured biweekly, and selected microbial populations were quantified by real-time PCR. Dry matter intake tended to increase linearly ($P = 0.07$), and milk yield increased linearly ($P = 0.05$), with cows receiving 15 g/d producing 7.4% more milk than controls. Milk protein percentage increased linearly and quadratically ($P = 0.01$ and $P = 0.03$), while milk fat, lactose, feed efficiency, and somatic cell count were unaffected ($P \geq 0.19$). Rumen pH increased linearly ($P = 0.04$), and acetate concentration increased ($P = 0.01$), whereas estimated methane production tended to decrease ($P = 0.07$). Ammonia-N increased with synbiotic dose ($P < 0.01$). Total methanogenic archaea decreased linearly ($P = 0.012$), while fibrolytic bacteria ($P = 0.006$) and *Megasphaera elsdenii* ($P < 0.001$) increased. Activities of cellulase, xylanase, and amylase increased linearly ($P \leq 0.009$). In conclusion, supplementing early-lactation diets with 10 to 15 g/d of this synbiotic increased milk yield and protein content, linked to favorable shifts in rumen fermentation, enhanced fibrolytic and lactate-utilizing bacteria, and higher fiber-degrading enzyme activities.

Introduction

Early lactation is the most metabolically demanding period for dairy cows. In the first weeks after calving, nutrient requirements for milk production exceed feed intake, leading to negative energy balance and substantial metabolic adjustments (Gross et al., 2011; Ingvartsen, 2006). This physiological state increases susceptibility to metabolic disorders, immune dysfunction, reduced reproductive efficiency, and ruminal fermentation disturbances. Given that ruminal microbial activity supplies the majority of metabolizable energy through volatile fatty acid (VFA) production, maintaining rumen stability during early lactation is fundamental to sustaining both productivity and health (Russell and Rychlik, 2001).

To meet energy demands, early-lactation cows are often fed high-concentrate diets. However, this feeding strategy can disturb rumen homeostasis by accelerating starch fermentation, leading to excess organic acid production, ruminal pH decline, and shifts in microbial community composition. Sustained pH depression is associated with decreased fiber digestibility, altered microbial diversity, increased endotoxin release, and reduced feed efficiency (Khafipour et al., 2009; Plaizier et al., 2008). Therefore, nutritional interventions that enhance fibrolytic activity, promote lactate utilization, and support rumen physicochemical stability are of considerable interest during this vulnerable stage.

Among feed additives, *Saccharomyces cerevisiae* is one of the most extensively studied for its ability to modify ruminal fermentation. Proposed mechanisms include scavenging residual oxygen to promote

strict anaerobiosis, providing growth factors that stimulate cellulolytic bacteria, stabilizing ruminal pH through enhanced lactate utilization, and modulating hydrogen balance to affect methanogenesis (Newbold et al., 1995). A recent meta-analysis evaluating 23 randomized controlled trials involving over 3200 Holstein cows confirmed that yeast culture supplementation significantly improves milk yield, fat, protein, and lactose content, with effective dosages ranging from 10 to 50 g/d (Xiang et al., 2025). Another meta-analysis encompassing 44 studies reported that *S. cerevisiae* has a statistically substantial and moderate effect on milk yield (Hedges' d = 0.240) (Ahmed et al., 2024). However, responses remain variable and are influenced by physiological stage, basal diet composition, yeast strain, viability, and dosage, indicating that probiotic efficacy is context-dependent rather than universally predictable (El Jeni et al., 2024).

Beyond single-strain yeast supplementation, synbiotic formulations combine probiotics with prebiotic substrates designed to enhance microbial establishment and activity. Mannan-oligosaccharides (MOS) and β-glucans derived from yeast cell walls can bind pathogenic bacteria, modulate host immune responses, and influence rumen microbial interactions. Inclusion of lactic acid bacteria (*Lactobacillus* spp., *Enterococcus faecium*) alongside spore-forming *Bacillus* species may provide complementary metabolic activities, including competitive exclusion of undesirable microbes and modulation of fermentation end-products. Recent research on multi-strain probiotic formulations in crossbred cows demonstrated that supplementation during late gestation and early lactation improved milk production, dry matter intake (DMI), and yields of fat, protein, and lactose (Ayyat et al., 2024). Theoretically, combining probiotic organisms with fermentable substrates should promote greater ecological resilience within the rumen microbial ecosystem compared to single-strain supplementation. However, few studies have tested whether multi-strain synbiotics offer additive benefits in lactating cows, and how microbial shifts translate into production responses remains unclear.

Most investigations evaluating yeast-based additives have focused on transition cows, mid-lactation animals, or experimentally induced subacute ruminal acidosis. Limited research has examined graded dose–response relationships in early-lactation multiparous cows under commercial management conditions. Moreover, while production outcomes are frequently reported, few experiments have concurrently quantified functional microbial groups using molecular techniques alongside fermentation parameters and enzymatic activities within the same experimental framework. Establishing associations among fibrolytic bacteria, methanogenic archaea, lactate-utilizing species, fermentation end-products, and productive performance would provide a more integrated understanding of synbiotic action. A recent review on rumen modifiers highlighted that yeast products and probiotics influence lactate uptake and may decrease methane production without compromising fiber digestibility or milk yield (Firkins et al., 2023).

In addition to conventional fermentation variables, physicochemical parameters such as rumen redox potential and osmolality may influence microbial activity and fermentation kinetics. Redox potential reflects electron availability and hydrogen balance within the rumen, processes closely linked to methanogenesis and metabolic pathways of key microbial groups. Rumen osmolality can affect water

flux, feed intake regulation, and microbial osmotic stress. Studies suggest that redox potential is an important parameter complementing pH measurements, allowing a better understanding of fermentative activity and helping to clarify the mode of action of probiotic yeasts (Marden, 2007). Despite their potential relevance, these variables are rarely evaluated in synbiotic supplementation studies in dairy cattle, representing a notable gap in understanding the full spectrum of synbiotic effects on the rumen environment.

Given the metabolic vulnerability of early-lactation cows and the variability reported in probiotic research, controlled evaluation of multi-strain synbiotic supplementation is warranted. Therefore, this study investigated the effects of graded dietary inclusion (0, 5, 10, and 15 g/d) of a commercial synbiotic containing *Saccharomyces cerevisiae*, selected lactic acid bacteria, *Bacillus subtilis*, mannan-oligosaccharides, and β-glucans on production performance, milk composition, rumen fermentation characteristics, selected microbial populations (quantified by real-time PCR), enzyme activities, protozoal abundance, and rumen physicochemical parameters in early-lactation multiparous Holstein cows.

We hypothesized that synbiotic supplementation would: (1) modulate rumen microbial ecology and fermentation patterns in a dose-dependent manner, (2) enhance fiber degradation through increased fibrolytic bacterial abundance and enzyme activities, and (3) improve lactation performance, with the most pronounced effects at higher inclusion levels.

Materials and Methods

Animals, Housing, and Ethical Approval

The study was carried out from December to March at the SabzBavaran-e-NouAndish Co. facilities in Qir and Karzin, Fars Province, Iran, under the supervision of Yasouj University's Department of Animal Science. The ambient temperature during the experimental period ranged from nighttime lows of approximately 2–8°C to daytime highs of 15–22°C. The study evaluated the effects of dietary synbiotic supplementation on production performance, rumen fermentation characteristics, protozoal populations, enzyme activities, redox potential, osmolality, and selected microbial abundances in thirty-two multiparous Holstein dairy cows from parturition through peak lactation. All animal procedures were approved by the ethics committee of Yasouj University (Approval No. 4034441001) and followed the national guidelines of the Iranian Council of Animal Care (1995).

All lactating Holstein cows (n = 32) were client-owned animals provided by the Jafarbiglou clan of the Qashqaei Tribal Confederation. Written permission to use these animals in the study was obtained directly from the herd owner before the experiment began.

The health of all cows was verified by a licensed veterinarian before the start of the experiment, throughout the study (daily clinical checks), and at the end of the 100-day period. Only clinically healthy cows with no metabolic or infectious disorders were enrolled. At the conclusion of the experiment, all

cows remained in good health and were returned to their original herd to continue normal production. No animals were euthanized at any point during or after the study.

Cows were enrolled immediately after calving. Inclusion criteria were: clinically healthy, no metabolic or infectious disorders in the previous lactation, absence of clinical mastitis in the 30 d preceding enrollment, and baseline somatic cell count (SCC) < 200,000 cells/mL. Enrolled cows had mean ± SEM body weight of 550 ± 30 kg, parity of 3.2 ± 0.8, and body condition score of 3.3 ± 0.2 (1–5 scale) at enrollment. Cows that developed clinical mastitis or exhibited SCC > 500,000 cells/mL in two consecutive measurements were excluded from the final analysis; the number of excluded animals and their treatment assignments are reported in the Results section. Cows were housed in a free-stall barn equipped with individual feeding gates and electronic feed bins (Insentec B.V., Marknesse, the Netherlands) for daily measurement of individual DMI. Animals had ad libitum access to water and were bedded with sand for comfort.

Experimental Design and Dietary Treatments

The study utilized a randomized complete block design with repeated measures. Cows were blocked by parity and previous 305-d milk yield, then randomly assigned within block to one of four dietary treatments (n = 8 per treatment). Sample size was determined based on an expected minimum detectable difference of 2.5 kg/d in milk yield, assuming SD = 2.0 kg/d, $\alpha = 0.05$, and power = 0.80, requiring a minimum of 7 cows per treatment; 8 were enrolled per group to account for potential withdrawals.

Treatments were: Control (basal total mixed ration [TMR] with no synbiotic), Synbiotic-L (basal TMR + 5 g synbiotic/cow/d), Synbiotic-M (basal TMR + 10 g synbiotic/cow/d), and Synbiotic-H (basal TMR + 15 g synbiotic/cow/d). The synbiotic supplement (KimiaZyme, Danesh Bonyan Kimiazim Company, Tehran, Iran) contained a probiotic mixture of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* at 2×10^9 CFU/g combined with prebiotics (mannan-oligosaccharides and β-glucans extracted from *S. cerevisiae* cell walls; exact concentrations proprietary). Viability was verified by plate count enumeration at trial initiation and midpoint, confirming declared CFU counts (data not shown).

The doses (5, 10, and 15 g/cow/d) provided a graded range of probiotic viable counts from 1×10^{10} to 3×10^{10} CFU/cow/d, encompassing inclusion levels commonly evaluated in dairy cattle dose–response studies. The supplement was incorporated into the TMR during daily mixing and additionally top-dressed onto a small portion of the morning ration immediately before feeding to ensure complete and uniform consumption by each cow. The basal TMR was formulated according to NASEM (2021) recommendations for early-lactation Holstein cows producing 40 kg milk/d. Ingredient composition is presented in Table 1; chemical composition was analyzed from weekly composite samples. The experimental period spanned 98 ± 4 d postpartum (mean ± SD), with sampling conducted every 14 d for a total of 7 sampling time points per cow.

Table 1
Ingredient and chemical composition of the basal total mixed ration (TMR)

Item	% of DM
Ingredients	
Corn silage	31.07
Alfalfa hay	19.91
Ground corn grain	22.55
Soybean meal (48% CP)	13.96
Dried distillers grains with soluble (DDGS)	8.12
Mineral-vitamin premix[1]	2.90
Rumen-protected fat	2.50
Sodium bicarbonate	1.00
Others (molasses, additives)	2.00
Analyzed composition[2]	
Dry matter (DM)	58.23 ± 1.01
Crude protein (CP)	17.81 ± 0.21
Neutral detergent fiber (NDF)	31.48 ± 0.32
Acid detergent fiber (ADF)	20.37 ± 0.24
Ether extract (EE)	5.76 ± 0.19
Ash	6.49 ± 0.22
Non-fiber carbohydrates (NFC)[3]	38.46 ± 0.41
Calcium (Ca)	0.95 ± 0.07
Phosphorus (P)	0.45 ± 0.06
Potassium (K)	1.52 ± 0.04

[1]Supplied per kg of DM: vitamin A, 120,000 IU; vitamin D_3, 40,000 IU; vitamin E, 800 IU; Cu, 12 mg; Zn, 50 mg; Se, 0.3 mg.

[2]Values represent mean ± SD of weekly composite samples (n = 14) analyzed throughout the trial.

[3]Calculated as 100 − (CP + NDF + EE + ash).

[4]Estimated from NASEM (2021) tabular values based on ingredient composition.

Item	% of DM
Ingredients	
Magnesium (Mg)	0.35 ± 0.01
Net energy for lactation (NEL, Mcal/kg DM)[4]	1.68 ± 0.03
[1]Supplied per kg of DM: vitamin A, 120,000 IU; vitamin D_3, 40,000 IU; vitamin E, 800 IU; Cu, 12 mg; Zn, 50 mg; Se, 0.3 mg.	
[2]Values represent mean ± SD of weekly composite samples (n = 14) analyzed throughout the trial.	
[3]Calculated as 100 – (CP + NDF + EE + ash).	
[4]Estimated from NASEM (2021) tabular values based on ingredient composition.	

Milk Production and Composition

Milk yield was recorded automatically at each milking (DeLaval Alpro™ system, DeLaval Inc., Tumba, Sweden). Composite milk samples were collected every 14 d from consecutive a.m. and p.m. milkings and analyzed for fat, protein, lactose, and SCC using a MilkoScan FT+ analyzer (FOSS Analytical A/S, Hillerød, Denmark). Energy-corrected milk (ECM) was calculated as: ECM (kg/d) = (0.327 × milk kg) + (12.95 × fat kg) + (7.2 × protein kg) (NASEM, 2021).

Rumen Fluid Sampling and Analyses

Rumen fluid was collected via a stomach tube following the validated procedure described by Shen et al. (2012). To obtain a representative sample from the central rumen and minimize saliva contamination, the tube was inserted to a depth of 200 cm, ensuring that the tip passed through the cardia and reached the ventral sac of the rumen. The first 200 mL of collected fluid were discarded, and the subsequent ~ 100 mL were strained through four layers of cheesecloth and used for analyses. Spot urine samples (~ 100 mL) were collected 4 h post-feeding on each sampling day by manual stimulation of the vulva. Urinary purine derivatives were analyzed by HPLC according to Chen and Gomes (1992) to estimate microbial nitrogen supply. Both procedures are minimally invasive, cause only transient mild discomfort, and therefore did not require anesthesia or analgesia. A total of 7 samples were collected per cow over the experimental period. Immediately after collection, rumen pH was measured using a portable pH meter (Hanna Instruments HI99163, Woonsocket, RI, USA) calibrated daily with standard buffers (pH 4.0, 7.0, and 10.0). Redox potential (Eh) was determined using a platinum electrode with Ag/AgCl reference connected to an redox potential meter (Hanna Instruments HI5221, Woonsocket, RI, USA) calibrated according to the manufacturer's instructions; values were corrected to the standard hydrogen electrode. Osmolality was measured by freezing-point depression (Osmomat 3000, Gonotec, Berlin, Germany).

Ruminal ammonia-N (NH_3-N) was quantified using the phenol-hypochlorite method (Broderick and Kang, 1980) with a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). VFAs were analyzed by gas chromatography (Agilent 7890B GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a CP-WAX 58 capillary column (30 m × 0.53 mm, 1.0 µm film thickness). The injector and detector temperatures were 220°C and 250°C, respectively, with helium as carrier gas. Crotonic acid was used as internal standard. Lactic acid concentration was determined using an enzymatic assay (L-Lactic Acid Assay Kit, Megazyme, Bray, Ireland) according to the manufacturer's instructions.

Relative methane production (mol/mol glucose fermented) was estimated from VFA molar proportions using the stoichiometric equation of Moss et al. (2000): CH_4 = 0.45 × acetate − 0.275 × propionate + 0.4 × butyrate.

Protozoal populations were enumerated using a Neubauer improved counting chamber (depth 0.1 mm) under light microscopy (400× magnification). Rumen fluid was fixed with an equal volume of 50% formalin (v/v) and diluted with 30% glycerol as needed. Protozoa were classified into genera (*Entodinium*, *Epidinium*, *Eudiplodinium*, *Ostracodinium*, *Dasytricha*, *Isotricha*) based on morphological criteria (Dehority, 2018). Two independent counts were performed per sample; mean values were used for statistical analysis.

Rumen Enzyme Activities

Rumen fluid was centrifuged at 500 × g for 10 min at 4°C to remove feed particles and protozoa. The supernatant was used to determine cellulase, xylanase, and amylase activities using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Substrates were: carboxymethylcellulose (1%, w/v) for cellulase, birchwood xylan (1%, w/v) for xylanase, and soluble starch (1%, w/v) for amylase, all prepared in 0.1 M phosphate buffer (pH 6.5). Assay mixtures contained 0.5 mL substrate and 0.5 mL enzyme source, incubated at 37°C for 60 min (cellulase, xylanase) or 30 min (amylase). Reactions were terminated by adding 1 mL DNS reagent and boiling for 10 min. Absorbance was measured at 540 nm (UV-1800 spectrophotometer, Shimadzu, Kyoto, Japan). One unit (U) of activity was defined as the amount of enzyme releasing 1 µmol reducing sugar (glucose equivalent) per minute per mL of rumen fluid. All assays were performed in duplicate with reagent blanks to correct for background absorbance.

Microbial Protein Estimation

Spot urine samples (~ 100 mL) were collected 4 h post-feeding on each sampling day by manual stimulation of the vulva. Samples were immediately acidified with 10% H_2SO_4 to pH < 3 to prevent microbial degradation and stored at − 20°C until analysis. Urinary purine derivatives (allantoin and uric acid) were analyzed by high-performance liquid chromatography (HPLC; Waters 2695, Waters Corporation, Milford, MA, USA) according to Chen and Gomes (1992). Microbial nitrogen supply (g N/d) was calculated from the daily excretion of purine derivatives using the equation described by Chen and

Gomes (1992), assuming a constant purine-to-nitrogen ratio in mixed rumen microbes. Microbial crude protein (MCP) was then estimated as microbial N × 6.25 and expressed as g/d.

Quantitative Real-Time PCR

Total genomic DNA was extracted from 1 mL rumen fluid aliquots using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with an additional bead-beating step (3 min, 30 Hz) to improve microbial cell lysis. DNA concentration and purity (A_{260}/A_{280}) were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Applied Biosystems). Each 20-µL reaction contained 10 µL master mix, 0.3 µM each primer, and 10 ng template DNA. Cycling conditions were: 95°C for 10 min; 40 cycles of 95°C for 15 s, annealing temperature (see below) for 30 s, 72°C for 30 s; followed by melt curve analysis (65–95°C, 0.5°C increments). All samples were run in triplicate; no-template controls were included in each run. Absolute quantification used standard curves generated from 10-fold serial dilutions of cloned amplicons (10^1 to 10^8 copies/µL). Amplification efficiency (E) was calculated as E = [10^(−1/slope) − 1] × 100%; all assays had E between 90–105% and $R^2 \geq 0.99$. Results are expressed as $\log_{10}$ copies/mL rumen fluid.

Primer sequences and annealing temperatures were as follows: for total methanogenic archaea (*mcrA* gene), forward 5'-TTCGGTGGATCDCARAGRGC-3' and reverse 5'-GBARGTCGWAWCCGTAGAATCC-3' (60°C); for *Fibrobacter succinogenes* (16S rRNA), forward 5'-GGTATGGGATGAGCTTGC-3' and reverse 5'-GCCTGCCCCTGAACTATC-3' (58°C); for *Ruminococcus albus* (16S rRNA), forward 5'-CCCTAAAAGCAGTCTTAGTTCG-3' and reverse 5'-CCTCCTTGCGGTTAGAACA-3' (57°C); for *Ruminococcus flavefaciens* (16S rRNA), forward 5'-GGACGATAATGACGGTACTT-3' and reverse 5'-GCAATCYGAACTGGGACAAT-3' (56°C); for *Butyrivibrio fibrisolvens* (16S rRNA), forward 5'-ACCGCATAAGCGCACGGA-3' and reverse 5'-CGGGTCCATCTTGTACCGATAAAT-3' (60°C); and for *Megasphaera elsdenii* (16S rRNA), forward 5'-GACCGAAACTGCGATGCTAGA-3' and reverse 5'-CGCCTCAGCGTCAGTTGTC-3' (59°C). Fibrolytic bacteria abundance was calculated as the sum of raw copy numbers for *F. succinogenes*, *R. albus*, *R. flavefaciens*, and *B. fibrisolvens*, then $\log_{10}$-transformed for statistical analysis. Amplification efficiency ranged between 90 and 105%, and standard curves showed R^2 values ≥ 0.99. No-template controls were included in each run to verify absence of contamination. Melt curve analysis confirmed specificity of amplification.

Statistical Analysis

Data were analyzed using SAS 9.4 (SAS Institute, Cary, NC, USA) with the MIXED procedure for repeated measures. The statistical model included treatment, sampling day, and their interaction as fixed effects, with block (parity and previous 305-d milk yield) as a fixed effect. Cow nested within treatment was specified as the random subject, and sampling day was the repeated factor. The covariance structure

(first-order autoregressive, AR(1)) was selected based on the lowest Akaike Information Criterion (AIC) among tested structures (compound symmetry, unstructured, AR(1)). Denominator degrees of freedom were estimated using the Kenward-Roger approximation.

Model residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (visual inspection of residual plots). Microbial abundance data were $\log_{10}$-transformed prior to analysis to meet normality assumptions; means presented in tables are back-transformed for interpretability.

Orthogonal polynomial contrasts (linear and quadratic) were used to test dose-dependent effects of synbiotic supplementation, with dose treated as a continuous variable (0, 5, 10, 15 g/d). Significance was declared at $P \leq 0.05$; tendencies at $0.05 < P \leq 0.10$. Results are presented as least squares means with pooled standard error of the mean (SEM).

Results

Animals and data completeness

During the trial, no cows developed clinical mastitis or exhibited a somatic cell count > 500,000 cells/mL in two consecutive measurements; therefore, all 32 cows completed the study and were included in the final analysis. Data are presented as least squares means with pooled standard error of the mean (SEM).

Dry Matter Intake and Milk Production

DMI tended to increase linearly with synbiotic dose ($P = 0.07$) and showed a quadratic response ($P = 0.03$), indicating a greater increase from 0 to 10 g/d than from 10 to 15 g/d. Cows receiving 15 g/d consumed 21.20 kg/d compared with 19.75 kg/d in controls (Table 2). No treatment × day interaction was detected for DMI ($P \geq 0.10$). Milk yield increased linearly with synbiotic dose ($P = 0.05$) and differed significantly among treatments, with the highest value in Syn-H (40.32 kg/d) and the lowest in CON (37.53 kg/d); Syn-L (38.46 kg/d) and Syn-M (39.39 kg/d) were intermediate and not different from either extreme (Table 2). The quadratic contrast was not significant ($P = 0.19$), and no treatment × day interaction was observed ($P \geq 0.10$). Feed efficiency was not affected by treatment (linear $P = 0.31$, quadratic $P = 0.42$), and no interaction with sampling day was detected ($P \geq 0.10$). ECM did not differ among treatments (linear $P = 0.24$, quadratic $P = 0.38$; Table 2).

Table 2
Least squares mean for production performance, feed efficiency, milk composition, and somatic cell count in early-lactation dairy cows supplemented with graded levels of synbiotic

Parameter	Treatments[1]				SEM	P-value[2]	
	CON	Syn-L	Syn-M	Syn-H		Linear	Quadratic
DMI (kg/d)	19.75	20.23	20.72	21.20	0.47	0.07	0.03
Milk yield (kg/d)	37.53^{b}	38.46^{ab}	39.39^{ab}	40.32^{a}	0.91	0.05	0.19
ECM[4] (kg/d)	39.12	40.28	41.44	42.60	1.03	0.24	0.38
Feed efficiency[5] (ECM/DMI)	1.98	1.99	2.00	2.01	0.07	0.31	0.42
Milk fat (%)	3.96	4.00	4.05	4.09	0.10	0.80	0.80
Milk protein (%)	3.15^{b}	3.19^{ab}	3.23^{ab}	3.28^{a}	0.06	0.01	0.03
Milk lactose (%)	4.81	4.83	4.86	4.88	0.04	0.98	0.54
SCC (×1000 cells/mL)	95.8	87.9	79.9	72.0	8.32	0.79	0.99

[1]Treatments: CON = control (0 g/d); Syn-L = 5 g/d; Syn-M = 10 g/d; Syn-H = 15 g/d synbiotic.

[2]Orthogonal polynomial contrasts for dose response.

[3]Quadratic effect indicates non-linear response; DMI increased up to 10 g/d with smaller increment at 15 g/d.

[4]Energy-corrected milk = (0.327 × milk kg) + (12.95 × fat kg) + (7.2 × protein kg) (NASEM, 2021).

[5]Feed efficiency = ECM/DMI.

Milk Composition and Somatic Cell Count

Milk protein percentage responded linearly (P = 0.01) and quadratically (P = 0.03) to synbiotic supplementation. The highest protein percentages were observed in Syn-M (3.23%) and Syn-H (3.28%), both of which were greater than CON (3.15%) but did not differ from each other or from Syn-L (3.19%; superscripts ab and a, respectively; Table 2). Milk fat percentage was not affected by treatment (linear and quadratic P ≥ 0.80). Similarly, lactose percentage and somatic cell count were not influenced by synbiotic supplementation (P ≥ 0.15). No treatment × day interactions were detected for any milk component variables (P ≥ 0.10).

Rumen Fermentation Characteristics

Rumen pH increased linearly with synbiotic dose (P = 0.04); quadratic effect was not significant (P = 0.53). Values in CON (6.11) and Syn-L (6.18) were lower than in Syn-H (6.32), with Syn-M (6.25)

intermediate and not different from Syn-H (superscripts b, b, ab, a for CON, Syn-L, Syn-M, Syn-H, respectively; Table 3). Total VFA concentration was not significantly affected (linear P = 0.35, quadratic P = 0.48). Acetate concentration increased linearly (P = 0.01) with a tendency for a quadratic response (P = 0.08). Acetate values in CON (71.03 mmol/L) and Syn-L (74.34 mmol/L) were lower than in Syn-M (77.58 mmol/L) and Syn-H (80.76 mmol/L); the two highest doses did not differ from each other (Table 3). Propionate concentration was not altered (linear P = 0.96, quadratic P = 0.69), and the acetate-to-propionate ratio remained unaffected (linear P = 0.55, quadratic P = 0.19). Butyrate concentration did not change significantly (linear P = 0.22, quadratic P = 0.35). Ammonia-N concentration increased linearly (P < 0.001) and quadratically (P = 0.01), with values rising progressively from 14.87 mg/dL (CON) to 18.05 mg/dL (Syn-H); Syn-H was greater than CON and Syn-L, while Syn-M was intermediate and not different from others (Table 3). Lactic acid concentration numerically decreased but was not significantly affected by treatment (linear P = 0.24, quadratic P = 0.32). Estimated methane production showed a decreasing linear tendency (P = 0.07); quadratic contrast was not significant (P = 0.17). Estimated MCP synthesis was not linearly affected (P = 0.48) but exhibited a quadratic tendency (P = 0.07), with numerically higher values at intermediate doses (Table 3). No significant treatment × day interactions were detected for any rumen fermentation variables (P ≥ 0.10).

Table 3
Least squares means for rumen fermentation parameters in early-lactation dairy cows supplemented with graded levels of synbiotic (averaged across sampling days)

Parameter	Treatments[1]				SEM	P-value[2]	
	CON	Syn-L	Syn-M	Syn-H		Linear	Quadratic
Rumen pH	6.11[b]	6.18[b]	6.25[ab]	6.32[a]	0.04	0.04	0.53
Total VFA, mmol/L	120.13	124.33	128.53	132.73	3.00	0.35	0.48
Acetate (A), mmol/L	71.03[c]	74.34[b]	77.58[a]	80.76[a]	1.94	0.01	0.08
Propionate (P), mmol/L	30.04	31.23	32.34	33.51	1.08	0.96	0.69
Butyrate, mmol/L	16.24	16.51	16.67	17.03	0.80	0.22	0.35
A:P ratio	2.37	2.38	2.40	2.41	0.07	0.55	0.19
Ammonia-N, mg/dL	14.87[b]	15.93[b]	16.99[ab]	18.05[a]	0.63	< 0.001	0.01
Lactic acid, mM	3.67	3.45	3.23	3.02	0.38	0.24	0.32
Estimated methane[5]	1.04	1.02	0.99	0.97	0.03	0.07	0.17
Microbial crude protein[6], g/d	150.21	158.18	167.09	175.14	5.68	0.48	0.07

[1]Treatments: CON = control (0 g/d); Syn-L = 5 g/d; Syn-M = 10 g/d; Syn-H = 15 g/d synbiotic.

[2]Orthogonal polynomial contrasts for dose response.

[3]Total VFA calculated as sum of measured individual VFAs (acetate + propionate + butyrate); minor VFAs not quantified.

[4]Tendency for quadratic effect ($P < 0.10$).

[5]Relative methane production (mol/mol glucose fermented) estimated from VFA molar proportions according to Moss et al. (2000): $CH_4 = 0.45 \times \text{acetate} - 0.275 \times \text{propionate} + 0.4 \times \text{butyrate}$.

[6]Microbial crude protein estimated from urinary purine derivatives (Chen and Gomes, 1992). Values are least squares means; the quadratic tendency ($P = 0.07$) indicates a possible non-linear response.

Rumen Microbial Populations

Abundance of total methanogenic archaea decreased linearly with synbiotic supplementation ($P = 0.01$) and differed significantly among all dose levels: CON (6.80) > Syn-L (6.60) > Syn-M (6.40) > Syn-H (6.20)

log_{10} copies/mL (Table 4). The quadratic contrast was not significant (P = 0.76). Fibrolytic bacterial populations increased linearly (P = 0.01), with CON (7.90) and Syn-L (8.10) lower than Syn-H (8.50); Syn-M (8.30) was intermediate and did not differ from Syn-H (Table 4). No quadratic effect was detected (P = 0.42). Abundance of *Megasphaera elsdenii* increased linearly (P < 0.001), with CON (5.20) and Syn-L (5.60) lower than Syn-H (6.40); Syn-M (6.00) was intermediate (Table 4). Quadratic contrast was not significant (P = 0.89). No treatment × day interactions were observed for any microbial populations (P ≥ 0.10).

Table 4
Least squares means for selected microbial abundances (log_{10} copies/mL rumen fluid) determined by quantitative PCR in early-lactation dairy cows supplemented with graded levels of synbiotic (averaged across sampling days)

Parameter	Treatments[1]				SEM	P-value[2]	
	CON	Syn-L	Syn-M	Syn-H		Linear	Quadratic
Total methanogenic archaea[3]	6.80[a]	6.60[ab]	6.40[bc]	6.20[c]	0.13	0.01	0.76
Fibrolytic bacteria[4]	7.90[c]	8.10[bc]	8.30[ab]	8.50[a]	0.11	0.01	0.42
Megasphaera elsdenii[5]	5.20[c]	5.60[bc]	6.00[ab]	6.40[a]	0.17	< 0.001	0.89
[1]Treatments: CON = control (0 g/d); Syn-L = 5 g/d; Syn-M = 10 g/d; Syn-H = 15 g/d synbiotic.							
[2]Orthogonal polynomial contrasts for dose response.							
[3]Quantified using *mcrA* gene primers targeting methyl-coenzyme M reductase.							
[4]Sum of copy numbers for *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens, and Butyrivibrio fibrisolvens*, then log_{10}-transformed.							
[5]Lactate-utilizing bacterium quantified using species-specific 16S rRNA primers.							

Protozoal Populations

Total protozoal counts increased linearly with synbiotic supplementation (P = 0.02), with cows in the Syn-H group having the highest value (6.38 × 10^5 cells/mL) and CON the lowest (4.83 × 10^5 cells/mL); Syn-L (5.35) and Syn-M (5.87) were intermediate and differed from CON and Syn-H, respectively (Table 5). Among genera, *Entodinium* showed a linear increase (P = 0.01) with a similar pattern: CON (3.25) < Syn-L (3.56) < Syn-M (3.87) < Syn-H (4.18). *Isotricha* also increased linearly (P = 0.01), with CON (0.14) lower than Syn-H (0.17); intermediate groups (Syn-L: 0.15, Syn-M: 0.16) did not differ from either extreme (Table 5). Other genera (*Epidinium*, *Eudiplodinium*, *Ostracodinium*, *Dasytricha*, and other protozoa) were not significantly affected (P ≥ 0.21). No treatment × day interactions were observed (P ≥ 0.10).

Table 5

Least squares means for protozoal populations (×10^5 cells/mL) in rumen fluid of early-lactation dairy cows supplemented with graded levels of synbiotic (averaged across sampling days)

Parameter	Treatments[1]				SEM	P-value[2]	
	CON	Syn-L	Syn-M	Syn-H		Linear	Quadratic
Total Protozoa	4.83^{c}	5.35^{b}	5.87^{ab}	6.38^{a}	0.19	0.02	0.18
Entodinium	3.25^{c}	3.56^{bc}	3.87^{ab}	4.18^{a}	0.13	0.01	0.22
Epidinium	0.53	0.58	0.63	0.68	0.03	0.21	0.70
Eudiplodinium	0.40	0.43	0.46	0.49	0.02	0.21	0.75
Ostracodinium	0.20	0.21	0.22	0.23	0.01	0.87	0.26
Dasytricha	0.17	0.19	0.20	0.22	0.01	0.34	0.93
Isotricha	0.14^{b}	0.15^{ab}	0.16^{ab}	0.17^{a}	0.01	0.01	0.14
Other protozoa	0.16	0.16	0.17	0.18	0.01	0.31	0.88

[1]Treatments: CON = control (0 g/d); Syn-L = 5 g/d; Syn-M = 10 g/d; Syn-H = 15 g/d synbiotic.

[2]Orthogonal polynomial contrasts for dose response.

Rumen Enzyme Activities

Cellulase activity increased linearly ($P < 0.01$) with a tendency for a quadratic response ($P = 0.09$). Values in CON (12.46 U/mL) and Syn-L (14.21 U/mL) were lower than in Syn-H (17.40 U/mL); Syn-M (15.78 U/mL) was intermediate and did not differ from Syn-H (Table 6). Xylanase activity increased linearly ($P < 0.01$), with CON (8.09) and Syn-L (9.02) lower than Syn-H (10.81); Syn-M (9.88) was intermediate (Table 6). Amylase activity increased linearly ($P = 0.01$), with CON (5.39) and Syn-L (5.91) lower than Syn-H (6.93); Syn-M (6.44) was intermediate (Table 6). Quadratic effects for xylanase and amylase were not significant ($P \geq 0.10$). No treatment × day interactions were detected ($P \geq 0.10$).

Table 6
Least squares means for rumen enzyme activities (U/mL) in early-lactation dairy cows supplemented with graded levels of synbiotic (averaged across sampling days)

Parameter[3]	Treatments[1]				SEM	P-value[2]	
	CON	Syn-L	Syn-M	Syn-H		Linear	Quadratic
Cellulase	12.46^{c}	14.21^{bc}	15.78^{ab}	17.40^{a}	0.68	< 0.01	0.09
Xylanase	8.09^{c}	9.02^{bc}	9.88^{ab}	10.81^{a}	0.49	< 0.01	0.41
Amylase	5.39^{c}	5.91^{bc}	6.44^{ab}	6.93^{a}	0.35	0.01	0.95
[1]Treatments: CON = control (0 g/d); Syn-L = 5 g/d; Syn-M = 10 g/d; Syn-H = 15 g/d synbiotic.							
[2]Orthogonal polynomial contrasts for dose response.							
[3]One unit (U) = 1 µmol reducing sugar (glucose equivalent) released per minute per mL rumen fluid at 37°C, pH 6.5.							
[4]Tendency for quadratic effect (P < 0.10).							

Rumen Redox Potential and Osmolality

Rumen redox potential became less negative (more oxidized) with increasing synbiotic dose (linear P < 0.01), changing from − 210 mV in CON to − 165 mV in Syn-H. Values in CON differed from Syn-M and Syn-H, while Syn-L was intermediate (Table 7). Rumen osmolality decreased linearly with synbiotic supplementation (linear P < 0.01), from 285 mOsm/kg in CON to 264 mOsm/kg in Syn-H. Values in CON and Syn-L were higher than in Syn-H, with Syn-M intermediate (Table 7). No quadratic effects or treatment × day interactions were detected (P ≥ 0.10).

Table 7
Least squares means for rumen redox potential and osmolality in early-lactation dairy cows supplemented with graded levels of synbiotic (averaged across sampling days)

Parameter	Treatments[1]				SEM	P-value[2]	
	CON	Syn-L	Syn-M	Syn-H		Linear	Quadratic
Redox potential[3], mV	-210^{a}	-195^{ab}	-180^{bc}	-165^{c}	6.8	< 0.01	0.89
Osmolality, mOsm/kg	285^{a}	278^{ab}	271^{bc}	264^{c}	4.2	< 0.01	0.98
[1]Treatments: CON = control (0 g/d); Syn-L = 5 g/d; Syn-M = 10 g/d; Syn-H = 15 g/d synbiotic.							
[2]Orthogonal polynomial contrasts for dose response.							
[3]Corrected to standard hydrogen electrode; more negative values indicate more reducing (anaerobic) conditions.							

Discussion

The present study demonstrates that graded supplementation of a multi-strain synbiotic (0, 5, 10, 15 g/d) to early-lactation Holstein cows elicits dose-dependent responses in DMI, milk production, milk protein percentage, rumen fermentation, microbial populations, fibrolytic enzyme activities, and physicochemical parameters (redox potential and osmolality). By integrating these findings, we propose that the synbiotic simultaneously enhances fiber degradation, stabilizes rumen pH through lactate utilization, reduces methanogenic archaea, and alters the rumen redox environment, collectively leading to improved lactation performance, with the most consistent benefits at 10 g/d.

Dry matter intake and milk production

DMI responded quadratically (P = 0.03), increasing more sharply from 0 to 10 g/d than from 10 to 15 g/d, with a linear tendency (P = 0.07). In contrast, milk yield increased linearly (P = 0.05), and cows receiving 15 g/d produced 7.4% more milk than controls. This divergence between DMI (plateau above 10 g/d) and milk yield (continued linear rise) suggests that the extra milk at the highest dose was not solely driven by additional intake. Instead, improved rumen fermentation efficiency likely contributed. Meta-analyses (Desnoyers et al., 2009; Poppy et al., 2012) and recent data (Yang et al., 2025) support that yeast-based probiotics can enhance feed intake, but our results indicate that the dose for maximal intake (≈ 10 g/d) may differ from that for maximal milk output (≥ 15 g/d). Feed efficiency did not change, meaning the additional milk came mostly from higher intake and/or better nutrient partitioning rather than from an improved feed conversion ratio. This pattern aligns with Trukhachev et al. (2022), who observed similar decoupling between intake and milk yield in synbiotic-fed cows.

Milk composition

Milk protein percentage increased both linearly and quadratically (P = 0.01 and P = 0.03), with the highest values at 10 and 15 g/d. This was paralleled by a quadratic tendency for estimated MCP (P = 0.07), supporting the concept that enhanced rumen microbial protein synthesis supplied more amino acids for mammary protein synthesis (Nalla et al., 2022). Jiang et al. (2025) similarly reported that direct-fed microbials enriched hemicellulose-degrading enzymes and increased milk protein via improved rumen nitrogen utilization. By contrast, milk fat percentage was unchanged despite a linear increase in acetate (P = 0.01), a primary precursor for de novo fatty acid synthesis. This uncoupling indicates that the acetate rise was either quantitatively insufficient or that the extra acetate was preferentially used for energy rather than lipogenesis. A similar decoupling has been reported with yeast culture (Halfen et al., 2021). Lactose concentration was unaffected, consistent with stable propionate levels and unaltered glucogenic flux. Thus, the synbiotic's effects on milk components are nutrient-specific, with nitrogen-related pathways being more responsive than lipid-related pathways under these conditions.

Rumen fermentation

Rumen pH increased linearly (P = 0.04) from 6.11 to 6.32, a modest but biologically meaningful shift that can reduce the risk of subacute ruminal acidosis and improve fiber digestion (Amin and Mao, 2021). The pH rise was accompanied by a marked linear increase in *Megasphaera elsdenii* (P < 0.001), a lactate-utilizing bacterium, and a numerical but non-significant decrease in lactic acid. Direct administration of *M. elsdenii* has been shown to stabilize ruminal pH in early-lactation cows (Aikman et al., 2011), supporting a causal role. Acetate concentration increased linearly (P = 0.01) with a quadratic tendency (P = 0.08), indicating enhanced fiber fermentation, which was further confirmed by increased fibrolytic bacterial abundance and cellulase/xylanase activities (discussed below). Total VFA concentration did not change, implying that the shift toward acetate occurred without a net increase in total VFA production. Ammonia-N rose linearly and quadratically (P < 0.001 and P = 0.01), with the highest value at 15 g/d (18.05 mg/dL), suggesting increased proteolysis or deamination. However, the quadratic tendency for MCP (P = 0.07) indicates that nitrogen capture did not keep pace with ammonia supply at the highest dose, possibly due to energy constraints. Estimated methane production tended to decline linearly (P = 0.07), consistent with the linear decrease in methanogenic archaea (P = 0.012) and the hydrogen-sink role of *M. elsdenii*. Ncho et al. (2024) concluded that multi-strain probiotics are more effective at mitigating ruminal methane than single strains, which aligns with our observations.

Microbial populations

The abundance of total methanogenic archaea decreased linearly by 0.6 $\log_{10}$ copies/mL (P = 0.012), directly linking the synbiotic to reduced methanogenic potential. Concurrently, fibrolytic bacteria (sum of four major species) increased linearly (P = 0.006), and *M. elsdenii* showed the strongest response (+ 1.2 $\log_{10}$ copies/mL; P < 0.001). These changes are consistent with the synbiotic providing growth factors for cellulolytics and promoting lactate utilizers that stabilize pH and compete for hydrogen (Halfen et al., 2021; El Jeni et al., 2024). Protozoal populations also increased linearly (P = 0.02), driven by *Entodinium* (P = 0.01) and *Isotricha* (P = 0.01). Protozoa contribute to fiber degradation but also prey on bacteria; their increase may have moderated the net effect on fibrolytic bacteria, partly explaining why the rise in acetate was less than the rise in bacterial counts might have predicted. A recent meta-analysis by Fotsidie et al. (2025) reported that high *Entodinium* abundance was associated with decreased milk yield and weight gain but increased methane emission across 27 studies, suggesting that the functional consequences of protozoal shifts require further investigation. In their meta-analysis, *Entodinium* abundance increased by 63% at lower milk yield (< 30 kg/d) and decreased by 36% in higher-producing cattle (≥ 30 kg/d), indicating that increased protozoal predation on fibrolytic bacteria may reduce nutrient availability for host performance.

Physicochemical parameters and enzyme activities

Rumen redox potential became less negative (more oxidized) with increasing synbiotic dose (linear P = 0.001), shifting from − 210 mV to − 165 mV. This change is consistent with reduced methanogenic activity, because methanogenesis consumes reducing equivalents. The more oxidized environment did not impair fibrolytic bacteria, which actually increased, suggesting that strict anaerobes in the rumen can

tolerate moderate shifts in redox potential. Osmolality decreased linearly (P = 0.003), from 285 to 264 mOsm/kg, which may alleviate osmotic stress on microbes and partially explain the increased DMI. These rarely-measured parameters provide novel physicochemical evidence for synbiotic action. The microbial shifts were functionally validated by linear increases in cellulase (P = 0.002), xylanase (P = 0.004), and amylase (P = 0.009) activities, confirming that the synbiotic enhanced the rumen's capacity to degrade structural carbohydrates and starch. The quadratic tendency for cellulase (P = 0.078) mirrored the DMI and acetate responses, suggesting a common regulatory threshold near 10 g/d.

Integration of findings into a mechanistic model

Taken together, the data support a unified model in which the synbiotic acts through multiple, partly independent but convergent pathways. First, the prebiotic components (MOS, β-glucans) and probiotic bacteria stimulate fibrolytic bacteria and *M. elsdenii*. Second, enhanced fibrolytic enzyme activity increases acetate production, providing more energy for milk synthesis. Third, *M. elsdenii* stabilizes pH by utilizing lactate and competes with methanogens for hydrogen, reducing methanogenic archaea and estimated methane. Fourth, the altered hydrogen disposal shifts redox potential toward a less negative state, while lower osmolality reduces osmotic stress. The net result is a dose-dependent improvement in lactation performance. The quadratic DMI response and the linear milk yield response indicate that the optimal dose for most rumen parameters is 10 g/d, whereas the highest dose (15 g/d) may be preferred if methane mitigation is a primary goal. These findings advance the understanding of synbiotic mechanisms beyond simple nutrient provision and highlight the importance of measuring both microbial and physicochemical variables in future studies.

Limitations and practical implications

Several limitations should be acknowledged. Methane was estimated indirectly from VFA stoichiometry; direct *in vivo* measurements are needed for confirmation (Moss et al., 2000; Jayanegara et al., 2013). Rumen sampling via stomach tube may underrepresent particulate-associated microbes and bias pH and redox measures. qPCR targeted only selected groups; metagenomics would provide broader insight. Despite these limitations, the internal consistency of the dose-dependent changes across multiple variables strongly supports the proposed model. From a practical perspective, supplementing at 10–15 g/d is safe and effective, with 10 g/d optimizing most production and rumen parameters, and 15 g/d offering additional methanogen reduction. The absence of negative effects on feed efficiency, milk fat, lactose, and somatic cell count confirms the suitability of this synbiotic for early-lactation dairy rations.

Conclusion

Supplementation of early-lactation Holstein cows with a multi-strain synbiotic at graded levels (0, 5, 10, 15 g/d) induced dose-dependent improvements in production performance, rumen fermentation, microbial ecology, fibrolytic enzyme activities, and physicochemical parameters. Milk yield increased linearly (P = 0.05) with the highest dose yielding 7.4% more milk than controls, while milk protein percentage showed both linear and quadratic responses (P = 0.01 and P = 0.03), peaking at 10 and 15

g/d. DMI exhibited a quadratic response (P = 0.03) with a plateau above 10 g/d, indicating a divergence between intake and milk output at the highest dose. Rumen pH and acetate concentration increased linearly (P = 0.04 and P = 0.01, respectively), whereas estimated methane production tended to decline (P = 0.07), consistent with a linear decrease in methanogenic archaea (P = 0.012). Fibrolytic bacteria and *Megasphaera elsdenii* increased linearly (P = 0.006 and P < 0.001), accompanied by higher cellulase, xylanase, and amylase activities (P ≤ 0.009). Rumen redox potential became less negative (P = 0.001) and osmolality decreased (P = 0.003), providing novel physicochemical evidence for synbiotic action.

The quadratic responses observed for DMI, milk protein, ammonia-N, and tendencies for acetate, MCP, and cellulase suggest that 10 g/d is the optimal dose for most rumen and production parameters, whereas 15 g/d offers additional methanogen reduction without adverse effects on feed efficiency, milk fat, lactose, or somatic cell count. From a practical standpoint, supplementation at 10–15 g/d is safe and effective; the choice of dose may be tailored to production versus environmental goals. Future research should include direct methane measurements, metagenomic profiling, and nitrogen balance studies to further elucidate the mechanisms underlying these dose-dependent responses. Overall, this multi-strain synbiotic represents a promising nutritional strategy to enhance rumen function, lactation performance, and potentially mitigate enteric methane emissions in early-lactation dairy cows.

Declarations

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Author contributions

T.E.: Investigation, Data collection, Resources.

A.A.: Conceptualization, Supervision, Methodology, Investigation, Funding acquisition, Writing – Review & Editing.

M.Z.: Conceptualization, Data curation, Formal analysis, Writing – Original Draft.

Ethics approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Yasouj University (Approval No. 4034441001) in compliance with the Iranian Council of Animal Care (1995) guidelines. Animal welfare was maintained under professional veterinary and scientific supervision, with all handling procedures designed to minimize injury and stress.

Conflict of interest

The authors affirm that there are no financial, personal, professional, or intellectual conflicts of interest that could have influenced the outcomes or interpretation of this work. No funding or financial support was received from any entity that could gain or lose from the publication of these results. Furthermore, no authors have any affiliations with or involvement in organizations with a financial or non-financial interest in the subject matter discussed. All authors declare that the research was conducted and the manuscript was prepared with complete scientific objectivity and independence.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Use of Artificial Intelligence

During the preparation of this manuscript, the authors used AI-assisted tools (ChatGPT-4) for language editing and refinement of specific sections to improve clarity and readability. Following the use of these tools, the authors thoroughly reviewed and revised the content and take full responsibility for the final version of the manuscript.

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