In Vitro Study of the Effects of Lipopolysaccharide on Rumen Fermentation (Postprint)

Authors: Zhiwen Tang, Jiang Linshu, Yang Liang, Kun Wang, Xiong Benhai

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Abstract

This study aimed to investigate the effects of lipopolysaccharide (LPS) on rumen fermentation using an in vitro approach. Four healthy Holstein dairy cows with similar body condition, fitted with permanent rumen fistulas, were used for rumen fluid collection. The experiment consisted of a control group (no LPS supplementation) and a treatment group (LPS supplementation at 100,000 EU/mL). Samples were collected at 2, 4, 8, 12, and 24 h of fermentation to determine fermentation fluid pH and concentrations of volatile fatty acids, ammonia nitrogen (NH3-N), and microbial protein. The results demonstrated that with prolonged fermentation time, fermentation fluid pH gradually decreased in both control and treatment groups, while concentrations of total volatile fatty acids, NH3-N, microbial protein, and contents of acetate, propionate, and butyrate progressively increased. No significant differences were observed between the control and treatment groups ($P > 0.05$). However, at 8 and 24 h, fermentation fluid pH in the treatment group exhibited a decreasing trend compared to the control group ($0.05 \leq P < 0.10$). Similarly, NH3-N concentration in the treatment group showed a decreasing trend at 4, 8, and 24 h compared to the control group ($0.05 \leq P < 0.10$). These findings indicate that under in vitro fermentation conditions, LPS supplementation tends to decrease fermentation fluid pH and NH3-N concentration, although this effect is not statistically significant.

Full Text

Effects of Lipopolysaccharide on Rumen Fermentation Investigated by *In Vitro* Method

TANG Zhiwen[1], JIANG Linshu[2], YANG Liang[1], WANG Kun[1], XIONG Benhai[1]*

[1]State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

[2]Beijing Key Laboratory for Dairy Cow Nutrition, Beijing University of Agriculture, Beijing 102206, China

Abstract

This experiment was conducted to examine the effects of lipopolysaccharide (LPS) on rumen fermentation using an *in vitro* method. Four healthy Holstein cows fitted with permanent ruminal cannulas were used as donors for rumen fluid collection. The experiment consisted of a control group (no LPS added) and an experimental group (LPS added at 100,000 EU/mL). Samples were collected after 2, 4, 8, 12, and 24 hours of fermentation to determine pH, volatile fatty acids (VFA), ammonia nitrogen (NH_3-N), and microbial protein (MCP) concentrations in the fermentation fluid. The results showed that as fermentation time increased, pH gradually decreased while total VFA, NH_3-N, MCP concentrations, and acetic, propionic, and butyric acid contents gradually increased in both groups, with no significant differences between control and experimental groups ($P > 0.05$). However, the experimental group exhibited a tendency toward lower pH at 8 and 24 hours ($0.05 \leq P < 0.10$) and lower NH_3-N concentrations at 4, 8, and 24 hours ($0.05 \leq P < 0.10$) compared to the control. These findings indicate that LPS supplementation tends to decrease fermentation fluid pH and NH_3-N concentration under *in vitro* conditions, though this effect is not statistically significant.

Keywords: lipopolysaccharide; cow; *in vitro* fermentation; rumen fermentation

Introduction

Subacute ruminal acidosis (SARA) is a common nutritional metabolic disease in dairy cows, typically triggered by high dietary concentrate ratios, insufficient quality forage, and stress responses. SARA is diagnosed when ruminal pH remains between 5.2 and 5.8 for more than 180 minutes. This condition severely compromises cow health and production performance by inducing other metabolic diseases, reducing feed intake and milk yield, and altering milk composition. Plaizier et al. reported that approximately 20% of high-producing dairy cows suffer from SARA, causing annual losses of $0.5–1 billion to the North American dairy industry. Therefore, deeper investigation into SARA pathogenesis is essential for protecting cow health and industry profitability.

Current theories on SARA pathogenesis include three main hypotheses: lactic acidosis, organic acidosis, and lipopolysaccharide (LPS) with histamine toxicity. LPS, also known as endotoxin, is a component of Gram-negative bacterial outer membranes that is released in large quantities during bacterial rapid growth or lysis. Under intensive production systems, SARA incidence has increased due to inadequate physically effective neutral detergent fiber or excessive concentrate feeding. During SARA, ruminal pH drops significantly while LPS concentration increases substantially, and high LPS levels further exacerbate SARA. Steele et

al. found that feeding high-grain diets to induce SARA damaged ruminal epithelium structure, likely due to combined effects of high LPS concentration and low pH. This epithelial damage impairs absorption of volatile fatty acids (VFA) and other organic acids, causing their accumulation in the rumen. Additionally, damaged epithelium increases LPS translocation into peripheral circulation, triggering systemic inflammatory responses. If ruminal inflammation occurs, VFA absorption is further suppressed, creating a vicious cycle of VFA accumulation and pH decline that worsens SARA.

However, existing research on LPS effects in SARA has focused on LPS-animal interactions, with few studies examining whether LPS directly affects ruminal microbial fermentation to influence SARA progression. Emmanuel et al. and Chin et al. found that low pH combined with high LPS concentration increased mucosal permeability, suggesting this interaction might also alter microbial cell membrane structure and activity, thereby affecting fermentation products. Due to numerous confounding factors *in vivo*, this study employed an *in vitro* method to investigate LPS effects on rumen fermentation.

1.1 Experimental Materials

The LPS used in this experiment was purchased from Sigma-Aldrich (USA) in 10 mg vials with an activity of 3,000,000 EU/mg.

1.2 Donor Animals and Management

Four healthy Holstein cows with similar body condition, fitted with permanent ruminal cannulas, were selected at Chengyuan Shenglong Dairy Farm in Beijing as rumen fluid donors. The cows were fed a conventional total mixed ration (TMR) with composition and nutrient levels shown in Table 1 . Feed was provided twice daily at 07:00 and 19:30, with free access to water and feed.

Table 1 Composition and nutrient levels of the TMR (DM basis) %

Item	Content
Ingredients	
Rice stalk	
Maize silage stalk	
Alfalfa	
Corn	
Wheat bran	
Soybean meal	
Cottonseed meal	
Limestone	
CaHPO_{4}	
NaCl	

Item	Content
NaHCO_{3}	
Premix	
Total	
Nutrient levels	
NEL/(MJ/kg)	
CP	
EE	
NDF	
Ca	

One kilogram of premix contained: VA 250,000 IU, VD 340,000 IU, VE 1

1.3.1 Artificial Saliva Preparation

Artificial saliva was prepared one day before the experiment according to Menke et al., continuously aerated with CO_{2} until use. The composition is shown in Table 2 . Stock solutions were prepared as follows: to 400 mL distilled water, sequentially add 100 L microelement solution A, 200 mL bicarbonate buffer B, 200 mL phosphate buffer C, 1 mL resazurin indicator, and 40 mL reduction solution. After thorough mixing, CO_{2} was continuously bubbled through until the solution changed from pink to colorless, with final pH adjusted to 6.80. The solution was preheated to 39°C before use.

Table 2 Composition of stock solutions of artificial saliva salt

Item	Reagent	Dosage
Microelement solution A	CaCl_{2}·2H_{2}O	13.2 g
	MnCl_{2}·4H_{2}O	10.0 g
	CoCl_{2}·6H_{2}O	1.0 g
	FeCl_{3}·6H_{2}O	8.0 g
	Volume	100 mL
Buffer bicarbonate B	NH_{4}HCO_{3}	4.0 g
	NaHCO_{3}	35.0 g
	Volume	1,000 mL
Phosphate buffer C	Na_{2}HPO_{4}	5.7 g
	KH_{2}PO_{4}	6.2 g
	MgSO_{4}·7H_{2}O	0.6 g
	Volume	1,000 mL
Resazurin indicator	Resazurin	0.1 g
	Volume	100 mL
Reduction solution	Na_{2}S·9H_{2}O	160 mg
	Volume	625 mg/100 mL

1.3.2 *In Vitro* Fermentation Fluid

Rumen fluid was collected 2 hours before morning feeding, with 500 mL obtained from each of the 4 cannulated cows. The fluid was filtered through 4 layers of cheesecloth and immediately transferred to pre-warmed (39°C) thermos flasks continuously flushed with CO_{2}. Appropriate amounts of digesta were collected from each cow into the flasks and rapidly transported to the laboratory. In the laboratory, 250 mL of rumen fluid from each cow was combined. The digesta was washed 3 times with 2 L artificial saliva, filtered through 4 layers of cheesecloth, and mixed with the rumen fluid to complete the fermentation fluid preparation. The *in vitro* fermentation fluid was maintained at 39°C in a water bath with continuous CO_{2} aeration until use.

1.3.3 Fermentation Substrate

The fermentation substrate consisted of steam-flaked corn, soybean meal, Chinese leymus, and alfalfa. Feed ingredients were dried at 65°C for 48 hours, ground to pass a 1 mm sieve, weighed according to substrate proportions, and mixed thoroughly. Substrate composition and nutrient levels are shown in Table 3 .

Table 3 Composition and nutrient levels of the substrate (DM basis) %

Ingredient	Content	Nutrient level	Content
Steam-pressed corn		DM	
Soybean meal		CP	
Alfalfa		NDF	
Chinese wildrye		NEL/(MJ/kg)	
CaHPO_{4}		Ca	
Premix			
Total			

1.3.4 Experimental Design

Numerous studies have shown that when dairy cows develop SARA, ruminal LPS concentrations typically range from 80,000–150,000 EU/mL. Therefore, this experiment used an LPS supplementation level of 100,000 EU/mL. A two-factor completely randomized design was employed with two treatments: control group (no LPS) and experimental group (LPS at 100,000 EU/mL), with fermentation times of 2, 4, 8, 12, and 24 hours. Each group had 25 fermentation bottles, with 5 bottles per time point. Following Menke et al.' s *in vitro* fermentation method, 0.5 g of fermentation substrate and 75 mL of *in vitro* fermentation fluid were added to each 100 mL fermentation bottle, incubated at 39°C.

1.4 Sample Collection and Analysis

Samples were collected at 2, 4, 8, 12, and 24 hours post-fermentation. Fermentation bottles were removed from the incubator and placed in ice water to stop fermentation, then pH was immediately measured using a pH meter. The fermentation fluid was filtered through 4 layers of cheesecloth and divided into two centrifuge tubes. To one tube, 25% metaphosphoric acid was added at an appropriate ratio, mixed thoroughly, and stored at -20°C for VFA and NH_{3}-N analysis. The other tube was centrifuged at 150 ×g for 15 minutes, and 8 mL of supernatant was collected for MCP determination. NH_{3}-N concentration was measured using the indophenol colorimetric method, VFA concentration was determined by gas chromatography with external standards, and MCP was quantified using the purine method. Dry matter, crude protein, ether extract, calcium, and phosphorus contents in diets and substrates were analyzed using conventional laboratory methods, while neutral detergent fiber was determined according to Van Soest et al. Net energy for lactation was calculated based on NY/T 34-2004.

1.5 Statistical Analysis

Data were initially processed using Excel 2016, then analyzed using the GLM procedure of SAS 9.2 software. Differences were considered significant at $P < 0.05$, and trends were identified at $0.05 \ P < 0.10$.

2.1 Fermentation Fluid pH

As shown in Table 4 , fermentation fluid pH in both control and experimental groups decreased with increasing fermentation time. No significant differences were observed between groups at any time point ($P > 0.05$), though the experimental group showed a decreasing trend compared to the control at 8 and 24 hours ($0.05 \ P < 0.10$).

Table 4 Effects of LPS supplementation on dynamic change of pH in *in vitro* rumen fermentation fluid

Item	Time/h	Control group	Experimental group	P-value
pH				

Values in the same row with different lowercase letter superscripts differ significantly ($P < 0.05$), while the same or no letter superscripts indicate no significant difference ($P > 0.05$). The same applies below.

2.2 Fermentation Fluid VFA Concentration

Table 5 shows that acetic, propionic, and butyric acid contents and total VFA (TVFA) concentration increased over time in both groups, with no significant differences between control and experimental groups at 2, 4, 8, 12, or 24 hours ($P > 0.05$). The acetic/propionic acid ratio initially increased then decreased, peaking at 12 hours in both groups, but showed no significant differences between groups at any time point ($P > 0.05$).

Table 5 Effects of LPS supplementation on dynamic change of VFA concentrations in *in vitro* rumen fermentation fluid

Item	Time/h	Control group	Experimental group	P-value
TVFA/(mmol/L)				
Acetic acid/%				
Propionic acid/%				
Butyric acid/%				
Acetic/propionic acid				

2.3 Fermentation Fluid NH_{3}-N Concentration

As presented in Table 6 , NH_{3}-N concentration increased gradually with fermentation time in both groups. No significant differences were found between control and experimental groups at any time point ($P > 0.05$), though the experimental group showed a decreasing trend at 4, 8, and 24 hours (0.05 $P < 0.10$).

Table 6 Effects of LPS supplementation on dynamic change of NH_{3}-N concentrations in *in vitro* rumen fermentation fluid (mg/dL)

Item	Time/h	Control group	Experimental group	P-value
NH_{3}-N				

2.4 Fermentation Fluid MCP Concentration

Table 7 demonstrates that MCP concentration increased over time in both groups, with no significant differences between control and experimental groups at any time point ($P > 0.05$).

Table 7 Effects of LPS supplementation on dynamic change of MCP concentrations in *in vitro* rumen fermentation fluid (mg/mL)

Item	Time/h	Control group	Experimental group	P-value
MCP				

3.1 Effects of LPS Supplementation on Dynamic pH Changes in *In Vitro* Rumen Fermentation Fluid

In vivo, ruminal pH is influenced by saliva secretion, organic acid production, absorption and excretion rates, and buffering substances. During *in vitro* fermentation, pH primarily depends on production of acidic substances like VFA and alkaline substances like NH_{3}-N. Zhang et al. observed decreasing pH with extended fermentation time in *in vitro* studies. Similarly, this experiment showed pH decreased over time in both groups, likely due to continuous VFA accumulation.

3.2 Effects of LPS Supplementation on Dynamic VFA Changes in *In Vitro* Rumen Fermentation Fluid

Acetic, propionic, and butyric acids are the main products of ruminal carbohydrate fermentation, accounting for approximately 95% of TVFA and serving as energy sources for animal production while maintaining rumen environment. In this study, TVFA and individual VFA concentrations increased gradually due to continuous production without absorption or removal in the sealed fermentation bottles, consistent with Zhang et al.' s findings. The acetic/propionic ratio initially increased then decreased, peaking at 12 hours. This pattern likely reflects high fiber content in early fermentation stages favoring acetate production, followed by a shift to propionate fermentation as fiber decreased during later stages.

3.3 Effects of LPS Supplementation on Dynamic NH_{3}-N Changes in *In Vitro* Rumen Fermentation Fluid

Ruminal NH_{3}-N originates from degradation of feed protein, non-protein nitrogen, and endogenous protein, serving as the primary substrate for MCP synthesis. Consistent with Zhang et al., this study showed increasing NH_{3}-N concentration over time, likely due to microbial degradation without ruminal wall absorption or digesta passage. However, Ouyang et al. reported NH_{3}-N initially increased then decreased in their *in vitro* study. The decreasing trend in NH_{3}-N observed in the experimental group at 4, 8, and 24 hours contrasts with Jing et al., who found increased ruminal NH_{3}-N after intravenous LPS infusion in dairy cows, possibly due to reduced gastrointestinal motility. Low pH can inhibit microbial growth and reduce fermentation rate, decreasing NH_{3}-N production. The trend toward lower pH in the experimental group at 8 and 24 hours may explain the observed NH_{3}-N reduction.

3.4 Effects of LPS Supplementation on Dynamic MCP Changes in *In Vitro* Rumen Fermentation Fluid

Ruminal MCP synthesis involves complex biological processes, providing high-quality nitrogen that meets 40–80% of animal protein requirements. MCP concentration increased over time in this study as carbohydrates and nitrogen

sources were gradually degraded, allowing microbes to synthesize MCP using NH_{3}-N. However, LPS supplementation did not significantly affect MCP concentration in fermentation fluid.

In conclusion, under *in vitro* fermentation conditions, LPS supplementation tends to decrease fermentation fluid pH and NH_{3}-N concentration, though these effects are not statistically significant.

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Note: Figure translations are in progress. See original paper for figures.