

Effects of *Lonicera japonica* Extract on In Vitro Rumen Fermentation Parameters and Gas Production: Postprint

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Abstract

This experiment aimed to investigate the effects of honeysuckle extract supplementation on in vitro rumen fermentation parameters and gas production. Four healthy Holstein dairy cows with similar body condition and permanent rumen fistulas were selected as experimental animals for rumen fluid collection. The experiment was divided into 5 groups: the control group received no honeysuckle extract in the fermentation substrate, while the experimental groups received 0.5, 1.0, 2.0, and 4.0 mg/g honeysuckle extract in the fermentation substrate, respectively, with 6 replicates per group and the experiment repeated for 3 batches. Gas production was recorded at 1.5, 3.0, 6.0, 12.0, and 24.0 h of fermentation, and rumen fermentation parameters were measured after 24.0 h of in vitro fermentation. The results showed: 1) Supplementation with 1.0, 2.0, and 4.0 mg/g honeysuckle extract significantly decreased fermentation broth pH and ammonia nitrogen (NH₃-N) concentration compared with the control group ($P < 0.05$), and significantly increased the concentrations of lactic acid, microbial crude protein (MCP), total volatile fatty acids (TVFA), and 24.0 h gas production compared with the control group ($P < 0.05$). 2) The fermentation broth pH and concentrations of NH₃-N, lactic acid, and TVFA in the 0.5 mg/g honeysuckle extract group showed no significant differences compared with the control group and the other three experimental groups ($P > 0.05$), with only the fermentation broth MCP concentration and 24.0 h gas production being significantly higher than those of the control group ($P < 0.05$). The results indicated that under in vitro conditions, honeysuckle extract supplementation could effectively regulate rumen microbial fermentation status, and considering overall economic benefits, supplementation with 1.0 mg/g honeysuckle extract was most appropriate.

Full Text

Effects of Honeysuckle Extract on Rumen in Vitro Fermentation Parameters and Gas Production

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Abstract

This experiment was conducted to investigate the effects of honeysuckle extract supplementation on rumen in vitro fermentation parameters and gas production. Four healthy Holstein cows fitted with permanent ruminal cannulas and similar body condition were selected as rumen fluid donors. The experiment was divided into five groups: a control group with no honeysuckle extract added to the fermentation substrate, and four treatment groups with honeysuckle extract added at 0.5, 1.0, 2.0, and 4.0 mg/g of substrate (dry matter basis). Each group had six replicates, and the experiment was repeated for three batches. Gas production was recorded at 1.5, 3.0, 6.0, 12.0, and 24.0 h of fermentation, and rumen fermentation parameters were measured after 24.0 h of in vitro fermentation. The results showed that: (1) supplementation with 1.0, 2.0, and 4.0 mg/g honeysuckle extract significantly decreased fermentation fluid pH and ammonia nitrogen (NH₃-N) concentration compared to the control group ($P < 0.05$), while significantly increasing lactic acid, microbial protein (MCP), and total volatile fatty acid (TVFA) concentrations as well as 24.0 h gas production ($P < 0.05$). (2) The 0.5 mg/g honeysuckle extract group showed no significant differences in pH, NH₃-N, lactic acid, or TVFA concentrations compared to the control or other treatment groups ($P > 0.05$), with only MCP concentration and 24.0 h gas production being significantly higher than the control ($P < 0.05$). These results indicate that honeysuckle extract can effectively regulate rumen microbial fermentation under in vitro conditions. Considering economic benefits, the optimal supplementation level is 1.0 mg/g.

Keywords: honeysuckle extract; rumen in vitro fermentation; dairy cow

Introduction

For decades, antibiotics have been used as feed additives in ruminant production to prevent energy metabolic diseases, regulate rumen fermentation, and improve animal performance. However, with increasing quality of life, concerns have grown regarding problems caused by antibiotic residues, such as carcinogenic, teratogenic, and mutagenic effects. In 2006, the European Union explicitly

banned antibiotic use in animal feed. Consequently, the search for antibiotic alternatives has become an increasingly important research focus worldwide.

Traditional Chinese herbal medicine is rich in proteins, carbohydrates, fats, and trace elements, making it suitable as a feed additive. Compared to other additives, traditional Chinese herbs are non-toxic, do not induce drug resistance, and leave no residues, ensuring the safety of animal products. However, relatively few Chinese herbs have been studied as feed additives for animal production. Therefore, new herbal additives that can effectively regulate rumen microecology and improve animal performance are highly anticipated.

Honeysuckle, also known as *Lonicera japonica* Thunb, is the dried flower bud of the Caprifoliaceae family. As a valuable traditional Chinese medicine, it is widely used in pharmaceuticals and health food products. Honeysuckle contains organic acids, flavonoids, saponins, volatile oils, and trace elements, with chlorogenic acid as its main active component. Research has demonstrated that honeysuckle possesses broad-spectrum antibacterial, antiviral, anti-inflammatory, immune-enhancing, and hepatoprotective effects. Honeysuckle and its extracts have gradually been applied in livestock and poultry production. Studies have shown that honeysuckle extract can improve antioxidant capacity and alleviate heat stress in beef cattle. Other research has found that honeysuckle can improve growth performance and intestinal microflora in broilers and act as a microecological regulator to adjust intestinal flora disorders in rats. Therefore, this study employed *in vitro* rumen fermentation techniques to investigate the regulatory effects of honeysuckle extract on dairy cow rumen microbial fermentation, providing a theoretical basis for its application as a feed additive in animal production.

Materials and Methods

1.1 Experimental Materials The honeysuckle extract used in this experiment was purchased from Shaanxi Zhongxin Biotechnology Co., Ltd., with a chlorogenic acid content of 10%.

1.2 Experimental Animals and Management Four healthy Holstein cows with similar body weight and good body condition, fitted with permanent ruminal cannulas, were selected as rumen fluid donors at Beijing Chengyuan Shenglong Dairy Farm. The cows were fed total mixed ration (TMR) consisting primarily of corn silage, alfalfa, steam-flaked corn, soybean meal, cottonseed meal, and dried distillers grains with solubles (concentrate-to-forage ratio of 60:40). Feeding occurred twice daily at 07:00 and 19:30, with free access to water and feed.

1.3.1 Artificial Saliva Preparation Artificial saliva salts were prepared one day before the experiment according to the method of Menke et al. The composition is shown in . After preparation, the solutions were combined in the following order: 400 mL distilled water, 100 L Solution A, 200 mL Solution B,

200 mL Solution C, 1 mL indicator, and 40 mL reducing solution. The mixture was continuously infused with CO₂ until the color changed from pink to colorless and transparent, and the pH was adjusted to 6.80. The solution was preheated to 39 °C before use.

1.3.2 In Vitro Fermentation Fluid Preparation Rumen fluid was collected 2.0 h before morning feeding, with 500 mL obtained from each of the four cannulated cows. The collected rumen fluid was filtered through four layers of cheesecloth and immediately transferred into a pre-warmed (39 °C) thermos flask infused with CO₂. Appropriate amounts of digesta were collected from each cow for microbial inoculation and rapidly transported to the laboratory. In the laboratory, 250 mL of rumen fluid from each cow was combined. The digesta were washed three times with 2 L of artificial saliva and filtered through four layers of cheesecloth before mixing with the rumen fluid to complete fermentation fluid preparation. The fermentation fluid was placed in a 39 °C water bath with continuous CO₂ infusion until use.

1.3.3 Fermentation Substrate Preparation The composition and nutrient levels of the fermentation substrate are shown in .

1.3.4 Experimental Design The in vitro rumen fermentation experiment followed the method of Cherdthong et al. Fermentation substrate ingredients were formulated as TMR, dried at 65 °C, and ground to pass through a 1 mm sieve. Approximately 0.2 g of substrate was accurately weighed into 100 mL glass syringes (coated with petroleum jelly to ensure airtightness). According to a randomized design, syringes were divided into five groups receiving honey-suckle extract at 0, 0.5, 1.0, 2.0, and 4.0 mg/g (substrate dry matter basis), with six replicates per group. The experiment was repeated for three batches. Each syringe was filled with pre-mixed fermentation fluid preheated to 39 °C, air was expelled, and the syringes were incubated at 39 °C with continuous shaking for 24.0 h.

1.3.5 Fermentation Fluid Sample Collection and Measurement Gas production was recorded at 1.5, 3.0, 6.0, 12.0, and 24.0 h of fermentation. After 24.0 h, samples were removed from the water bath and placed in ice water to stop incubation. pH was immediately measured using a portable pH meter (STARTER-300). The fermentation fluid was filtered through four layers of cheesecloth and divided into four centrifuge tubes. Three tubes were mixed with 25% metaphosphoric acid solution and stored at -20 °C for volatile fatty acid (VFA), ammonia nitrogen (NH₃-N), and lactic acid concentration determination. The remaining tube was centrifuged at 150×g for 15 min, and the supernatant was collected for microbial protein (MCP) concentration measurement.

NH₃-N concentration was determined using the indophenol method. The procedure was as follows: 0.05 mL sample was added to a test tube and mixed with

2.5 mL phenol solution; 2.0 mL sodium hypochlorite solution was added and mixed; the tube was placed in 95 °C water bath for 5 min, then 60 °C water bath for 10 min; after cooling, absorbance was measured at 630 nm using a Tiammei UV-2600 spectrophotometer.

Lactic acid concentration was determined using the p-hydroxybiphenyl colorimetric method. The procedure was: fermentation fluid (or lactic acid standard solution for standard curve) was centrifuged at 4,000 r/min for 10 min; 5 mL supernatant was taken and mixed with 0.5 mL 20% copper sulfate; 0.5 mL of this solution was slowly mixed with 6 mL pre-cooled concentrated sulfuric acid, placed in boiling water for 5 min, then immediately transferred to ice water; 0.125 mL 1.5% p-hydroxybiphenyl solution was added, mixed rapidly, incubated at 30 °C for 30 min, then boiled for 90 s, cooled in ice water to room temperature, and measured at 565 nm using a Tiammei UV-2600 spectrophotometer with distilled water as blank.

VFA concentration was determined by external standard method using an Agilent-7890B gas chromatograph with flame ionization detector, PEG-20M+H₃PO₄ glass packed column, column temperature 120 °C, detector temperature 220 °C, argon as carrier gas at 30 mL/min, hydrogen from generator at 30 mL/min, air flow 300 mL/min, and injection volume 2 L.

MCP concentration was determined using the purine method. For standard curve preparation: (1) 5, 15, 25, 35, 45, and 55 mg yeast RNA were weighed into 10 mL tubes with 2 mL 0.6 mol/L HClO₄, heated at 95 °C for 1.0 h and cooled; (2) 6 mL 28.5 mmol/L NH₄H₂PO₄ was added, heated at 95 °C for 15 min, cooled, and centrifuged at 3,000×g for 10 min at 4 °C; (3) 1.6 mL supernatant was mixed with 6 mL 0.2 mol/L NH₄H₂PO₄, pH adjusted to 2-3 with 85% phosphoric acid; (4) 3.8 mL of pH-adjusted solution was mixed with 0.2 mL 0.4 mol/L AgNO₃, stored overnight at 4 °C in darkness; (5) centrifuged at 3,000×g for 10 min at 4 °C, supernatant discarded, precipitate washed with 5 mL pH 2 distilled water, centrifuged again; (6) 5 mL 0.5 mol/L HCl was added to precipitate, mixed, heated at 95 °C for 30 min, centrifuged at 3,000×g for 10 min; (7) supernatant was diluted 40-fold with 0.5 mol/L HCl and measured spectrophotometrically using Tiammei UV-2600 with 0.5 mol/L HCl as reference.

For fermentation fluid MCP determination: (1) 8 mL fermentation fluid was centrifuged at 20,000×g for 20 min at 4 °C; supernatant was discarded, 2.104 mL 0.6 mol/L HClO₄ was added, heated at 95 °C for 1.0 h and cooled; (2) steps 2-6 from standard curve preparation were followed; (3) measured at 260 nm with 0.5 mol/L HCl as reference, RNA content was determined from standard curve; (4) MCP concentration was calculated using: Microbial protein nitrogen (mg/mL) = [RNA measured value (mg/mL) × 17.83% / 10%] × dilution factor; MCP (mg/mL) = Microbial protein nitrogen (mg/mL) × 6.25.

Diet and fermentation substrate dry matter, crude protein, crude fat, calcium, and phosphorus contents were analyzed using conventional laboratory methods. Neutral detergent fiber and acid detergent fiber contents were determined using

the method of Van Soest et al. Net energy for lactation was calculated according to *Feeding Standard of Dairy Cows* (NY/T 34-2004): Net energy for lactation = $0.5501 \times \text{digestible energy (MJ/kg)} - 0.395$.

1.4 Statistical Analysis Experimental data were initially processed using Excel 2016 and further analyzed using the Mixed model in SAS 9.4. Differences were considered significant at $P < 0.05$.

Results

2.1 Fermentation Fluid pH and Lactic Acid, $\text{NH}_3\text{-N}$, and MCP Concentrations As shown in , the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups had significantly lower fermentation fluid pH than the control group ($P < 0.05$), while $\text{NH}_3\text{-N}$ and lactic acid concentrations were significantly higher than the control group with no significant differences among these three groups ($P > 0.05$). The 0.5 mg/g honeysuckle extract group showed no significant differences in pH, lactic acid, or $\text{NH}_3\text{-N}$ concentrations compared to the control or other treatment groups ($P > 0.05$). MCP concentrations in all treatment groups were significantly higher than the control group ($P < 0.05$), with the 2.0 and 4.0 mg/g groups being significantly higher than the 0.5 and 1.0 mg/g groups ($P < 0.05$).

2.2 Fermentation Fluid VFA Concentrations As shown in , the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups had significantly higher total volatile fatty acid (TVFA) concentrations than the control group ($P < 0.05$), while the 0.5 mg/g group showed no significant differences compared to the control or other treatment groups ($P > 0.05$). No significant differences were observed among any groups in acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid concentrations, or acetic acid/propionic acid ratio ($P > 0.05$).

2.3 Dynamic Changes of in Vitro Gas Production As shown in , the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups had significantly higher gas production than the control group at 1.5, 3.0, 6.0, 12.0, and 24.0 h ($P < 0.05$), with no significant differences in total gas production among these three groups at 1.5, 6.0, 12.0, and 24.0 h ($P > 0.05$). The 0.5 mg/g honeysuckle extract group had significantly higher gas production than the control group only at 24.0 h ($P < 0.05$), with no significant differences at 1.5, 3.0, 6.0, and 12.0 h ($P > 0.05$).

Discussion

3.1 Effects of Honeysuckle Extract on Fermentation Fluid pH and Lactic Acid, $\text{NH}_3\text{-N}$, and MCP Concentrations Rumen fluid pH in ruminants is influenced by numerous factors including saliva secretion, rumen buffering substances, and the production, absorption, and excretion rates of organic acids. However, the in vitro fermentation system is not affected by saliva secretion or organic acid absorption and excretion rates. Therefore, the

production of alkaline substances (such as $\text{NH}_3\text{-N}$) and organic acids in the fermentation fluid becomes the main factor affecting pH. The significantly lower pH in the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups may be due to higher lactic acid and TVFA concentrations compared to the control group. Previous studies have shown that honeysuckle extract administration significantly increased Bifidobacterium and Lactobacillus populations in rat intestines. Similarly, research on broilers receiving Shuanghuanglian propolis oral solution (containing honeysuckle, Scutellaria, Forsythia, and propolis) reported significantly increased intestinal Bifidobacterium and Lactobacillus populations. In this experiment, the significantly higher lactic acid concentration in the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups may result from promoted growth of lactic acid-producing bacteria. These findings suggest that honeysuckle extract can function as a prebiotic to modulate animal intestinal microflora, promoting beneficial bacteria proliferation while inhibiting harmful bacteria growth. The significantly higher MCP concentrations in all treatment groups compared to the control indicate that honeysuckle extract may promote microbial growth, with promotion effects stronger than inhibition effects. $\text{NH}_3\text{-N}$ concentration reflects the comprehensive balance between feed nitrogen degradation and microbial $\text{NH}_3\text{-N}$ utilization in the rumen. In this study, the significantly higher $\text{NH}_3\text{-N}$ concentrations in the 1.0, 2.0, and 4.0 mg/g groups, accompanied by significantly higher MCP concentrations, suggest that the increased $\text{NH}_3\text{-N}$ resulted from enhanced microbial degradation efficiency of nitrogenous substances in the fermentation substrate rather than insufficient $\text{NH}_3\text{-N}$ utilization.

3.2 Effects of Honeysuckle Extract on Fermentation Fluid VFA Concentrations Volatile fatty acids produced by rumen fermentation account for approximately 70-80% of the energy absorbed by ruminants. Acetic acid, propionic acid, and butyric acid are the main products of rumen carbohydrate fermentation, comprising about 95% of TVFA, and primarily function to provide energy for animal production and maintain rumen environment. In this experiment, the significantly higher TVFA concentrations in the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups indicate that honeysuckle extract supplementation can promote greater VFA production by microbial fermentation, which is beneficial for providing more energy for animal production. The significantly increased TVFA concentrations in these three groups may be attributed to promoted proliferation of certain microorganisms by honeysuckle extract, leading to greater VFA production. This is supported by the significantly higher MCP concentrations observed in these groups.

3.3 Effects of Honeysuckle Extract on Dynamic Changes of in Vitro Gas Production Gas production is an important indicator for evaluating rumen microbial fermentation in vitro systems, comprehensively reflecting the fermentability of substrates, indicating the overall trend of rumen microbial fermentation activity, and serving as a comprehensive indicator of substrate availability. In this experiment, the significantly higher gas production at all

time points in the 1.0, 2.0, and 4.0 mg/g honeysuckle extract groups demonstrates that honeysuckle extract supplementation can improve rumen microbial utilization efficiency of fermentation substrates. The significantly higher MCP concentrations in these groups also indicate that honeysuckle extract promotes rumen microbial growth, which may explain the increased gas production.

Conclusion

Under in vitro culture conditions, supplementation with 1.0, 2.0, and 4.0 mg/g honeysuckle extract significantly decreased fermentation fluid pH and $\text{NH}_3\text{-N}$ concentration while significantly increasing lactic acid, MCP, and TVFA concentrations and gas production at 24.0 h. Considering economic benefits, the optimal supplementation level is 1.0 mg/g.

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