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Effects of Hard Kernel Oil on Rumen In Vitro Fermentation and Fatty Acid Composition (Post-print)

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Abstract

This study employed the in vitro gas production method to investigate the effects of dietary supplementation with 1%, 2%, 3%, and 4% (dry matter basis) of hard core oil on 24-h in vitro rumen fermentation gas production parameters, fermentation parameters, and fatty acid composition, aiming to determine the optimal supplementation level of hard core oil for rumen fermentation. The results indicated: 1) Dietary supplementation of hard core oil had no significant effect on 24-h gas production, theoretical maximum gas production, or gas production rate ($P>0.05$). 2) Ammonia nitrogen ($P=0.044$), total volatile fatty acids ($P<0.001$), acetate ($P<0.001$), propionate ($P=0.047$), and butyrate concentrations ($P=0.017$) exhibited a quadratic increase with increasing levels of hard core oil supplementation, with total volatile fatty acids and acetate concentrations in the 1%, 2%, and 3% groups being significantly higher than those in the control and 4% groups ($P<0.05$); pH, microbial protein concentration, dry matter degradability, neutral detergent fiber degradability, and acid detergent fiber degradability (ADFD) were not affected by hard core oil supplementation ($P>0.05$). 3) The content of saturated fatty acids exhibited a linear ($P=0.008$) and quadratic ($P=0.028$) decrease with increasing levels of hard core oil supplementation, with C18:0 content also showing a linear decrease ($P=0.030$); the content of unsaturated fatty acids exhibited a linear ($P=0.008$) and quadratic ($P=0.028$) increase with increasing levels of hard core oil supplementation, with t9-C18:1 content showing a linear increase ($P=0.002$), t11-C18:1 content showing a quadratic increase ($P<0.001$), and c9-C18:1 content showing both linear ($P=0.028$) and quadratic ($P=0.005$) increases, with both t9-C18:1 and c9-C18:1 contents reaching maximum values at the 3% supplementation level. Under the experimental conditions of this study, supplementation with hard core oil at 1%, 2%, and 3% levels increased the content of unsaturated fatty acids in

rumen in vitro fermentation while increasing the concentration of volatile fatty acids, with the 3% supplementation level demonstrating the optimal effect.

Full Text

Effects of Scleropyrum wallichianum Oil on in Vitro Rumen Fermentation Characteristics and Fatty Acid Composition

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Abstract

This study investigated the effects of supplementing diets with 1%, 2%, 3%, and 4% (dry matter basis) Scleropyrum wallichianum oil on 24 h gas production parameters, fermentation parameters, and fatty acid composition using an in vitro gas production method, aiming to determine the optimal supplementation level for rumen fermentation. The results showed that: (1) Dietary supplementation with Scleropyrum wallichianum oil had no significant effects on 24 h gas production, theoretical maximum gas production, or gas production rate ($P>0.05$). (2) Ammonia nitrogen ($P=0.044$), total volatile fatty acid ($P<0.001$), acetate ($P<0.001$), propionate ($P=0.047$), and butyrate concentrations ($P=0.017$) increased quadratically with increasing oil supplementation. Moreover, total volatile fatty acid and acetate concentrations in the 1%, 2%, and 3% groups were significantly higher than those in the control and 4% groups ($P<0.05$). pH, microbial protein concentration, dry matter degradation rate, neutral detergent fiber degradation rate, and acid detergent fiber degradation rate were not affected by oil supplementation ($P>0.05$). (3) Saturated fatty acid content decreased linearly ($P=0.008$) and quadratically ($P=0.028$) with increasing supplementation, with C18:0 content also decreasing linearly ($P=0.030$). Unsaturated fatty acid content increased linearly ($P=0.008$) and quadratically ($P=0.028$) with increasing supplementation. Specifically, t9-C18:1 content increased linearly ($P=0.002$), t11-C18:1 content increased quadratically ($P<0.001$), and c9-C18:1 content increased both linearly ($P=0.028$) and quadratically ($P=0.005$), with t9-C18:1 and c9-C18:1 reaching maximum values at the 3% supplementation level. Under the conditions of this experiment, supplementation with 1%,

2%, and 3% Scleropyrum wallichianum oil enhanced unsaturated fatty acid content and volatile fatty acid concentrations in in vitro rumen fermentation, with the 3% level showing the best effect.

Keywords: Scleropyrum wallichianum oil; gas production parameters; fermentation parameters; fatty acid composition

Introduction

The rumen is a natural anaerobic fermenter where energy loss occurs during fermentation [1]. To improve feed utilization efficiency and ruminant production performance, numerous studies have focused on developing modulators to regulate rumen fermentation processes. Plant oils contain active ingredients that can regulate rumen fermentation and are naturally non-toxic, leading to their widespread application in recent years. However, different plant oil types and supplementation levels produce varying effects, making it crucial to determine the optimal supplementation level for each oil type in ruminant production.

Research on plant oil modulation of rumen fermentation has primarily focused on gas production, fermentation parameters, and rumen fluid fatty acid composition. Zheng et al. [2] used in vitro gas production methods to demonstrate that supplementing diets with 2% and 4% cottonseed oil, soybean oil, rapeseed oil, corn oil, and sunflower oil (on a dry matter basis) significantly reduced gas production during rumen fermentation. Nanon et al. [3] found that adding cinnamon oil, clove oil, garlic oil, ginger oil, and lemongrass oil (200 mg, DM basis) to diets with a concentrate-to-forage ratio of 1:1 increased total volatile fatty acid (TVFA) concentration without significantly affecting the acetate-to-propionate ratio. Shi et al. [4] investigated the effects of perilla oil at supplementation levels of 1%, 2%, 3%, and 4% (DM basis) on in vitro rumen fermentation parameters, reporting no significant effects on pH or ammonia nitrogen (NH3-N) concentration, but significant reductions in dry matter degradation rate (DMD), neutral detergent fiber degradation rate (NDFD), and acid detergent fiber degradation rate (ADFD). Wang et al. [5], Liang et al. [6], and Zhu et al. [7] reported that supplementing diets with sunflower seed oil (7 mL/d), tea oil (4%, DM basis), and garlic oil (0.8 g/d), respectively, all increased the content of unsaturated fatty acids such as C18:1 and C18:2 in rumen fluid.

Scleropyrum wallichianum is a thorny small tree belonging to the family Santalaceae and genus *Scleropyrum*. As a native wild plant in Xishuangbanna, Yunnan Province, its kernels contain approximately 67% oil [8], comprising 11 fatty acids including C16:0, C12:0, C10:0, C20:0, C18:1, and C18:2, as well as uncommon alkyne acid components. Research has demonstrated that these alkyne acid components possess antimycobacterial and antiplasmodial properties [9]. The plant exhibits strong adaptability, is widely distributed in Xishuangbanna, and represents a substantial resource with relatively low production costs, indicating excellent development value and application prospects. This study employed in vitro gas production methods to investigate the effects of different supplementa-

tion levels of *Scleropyrum wallichianum* oil on rumen gas production parameters, fermentation parameters, and fatty acid composition, aiming to preliminarily determine the optimal dietary supplementation level and provide a theoretical basis for practical application in ruminant production.

Materials and Methods

1.1.1 Scleropyrum wallichianum Oil and Diet Samples

The *Scleropyrum wallichianum* oil used in this study was provided by the Yunnan Institute of Tropical Crops and produced from kernels via cold-chain pressing, resulting in a golden-yellow, clear, and transparent oil with 99.8% purity. Fatty acid composition was quantitatively determined after crude fat extraction using organic solvents, with results presented in Table 1 . The diet used for in vitro fermentation was identical to that fed to donor animals. The diet was air-dried and ground through a 2 mm sieve to serve as the fermentation substrate. Diet composition and nutrient levels are shown in Table 2 .

1.1.2 Rumen Fluid Collection and Buffer Preparation

The buffer solution used in the experiment was prepared according to the method of Menke et al. [10] and continuously infused with CO_2 while being maintained in a 39 °C water bath until use.

Three healthy Holstein dairy cows (third parity) fitted with permanent rumen fistulas were selected from the Changping Base of the Institute of Animal Science, Chinese Academy of Agricultural Sciences. The cows had an average body weight of $(550\$\pm50)kg, lactationdaysof(136\pm37)d, andmilkyieldof(25.3\pm2.79)kg.Ontheexperimentalday, run$ $warmed(39°C)thermosflasksfilledwithCO\{2\}, andimmediatelytransportedtothelaboratory.Thefluidwasfil$ infusion during filtration) and used immediately for the experiment.

1.2 Experimental Design

A completely randomized design was employed, consisting of a control group without supplementation and treatment groups with *Scleropyrum wallichianum* oil added at 1%, 2%, 3%, and 4% of substrate DM. Each treatment had five replicates. Fermentation was terminated after 24 h, and fermentation fluid and residual substrate were collected for determination of pH, NH_3-N concentration, microbial protein (MCP) concentration, volatile fatty acid (VFA) concentration, dry matter degradation rate (DMD), neutral detergent fiber degradation rate (NDFD), acid detergent fiber degradation rate (ADFD), and fatty acid content.

1.3 Experimental Procedures

The in vitro fermentation system consisted of an AGRS-III 64-channel automated gas production recording device and software system developed by China Agricultural University. Approximately 0.5 g of fermentation substrate was

weighed into 150 mL anaerobic fermentation bottles. Due to the adhesive nature of oil and the small supplementation amounts, directly weighing the oil would introduce substantial experimental error. Therefore, the required proportions were converted to volume and pipetted into the fermentation bottles. Each bottle containing 50 mL of buffer solution received 25 mL of filtered rumen fluid, was infused with CO_2 for 5 s, sealed, connected to the gas production sensors, and incubated at 39 °C for 24 h.

1.4 Sample Collection and Preparation

After 24 h of incubation, fermentation was terminated and fermentation fluid was immediately collected from the bottles. For VFA and NH_3-N analysis, 5 mL of fermentation fluid was collected in each of two 10 mL centrifuge tubes, and 1 mL of metaphosphoric acid solution was added to each. For MCP concentration and fatty acid analysis, 8 mL of fermentation fluid was collected in each of two additional 10 mL centrifuge tubes, centrifuged at 150×g for 15 min, and the supernatant was retained.

All fermentation fluid samples were stored at -20 °C. The remaining liquid and solid phases were thoroughly mixed, centrifuged at 4,000×g for 10 min, the supernatant was discarded, and all solid residues were collected for determination of DMD, NDFD, and ADFD.

1.5 Sample Analysis and Calculations

The pH of fermentation fluid was measured immediately after fermentation using a Seven GoTM precision pH meter. NH_3-N concentration was determined by the indophenol colorimetric method [11]. Five milliliters of metaphosphoric acid-treated fermentation fluid was centrifuged at 3,000×g for 10 min, and the supernatant was processed. Ammonium chloride was used to prepare standard solutions, and absorbance values of standards and samples were measured at 700 nm using a microplate reader (Thermo Electron Varioskan Flash, USA). NH_3-N concentration was calculated based on standard curves and absorbance values.

MCP concentration was determined by the purine method [12]. A standard curve was prepared using yeast RNA. Eight milliliters of fermentation fluid was centrifuged at 20,000×g for 20 min, processed, and analyzed by microplate reader at 260 nm. RNA values were calculated from absorbance values and the standard curve, and MCP concentration was calculated using the following formulas:

Microbial protein nitrogen (mg/mL) = RNA value × RNA nitrogen content (17.83%) / RNA nitrogen content in bacterial nitrogen (10%) × dilution factor; MCP (mg) = Microbial protein nitrogen (mg/mL) × 6.25 × fermentation fluid volume (mL).

VFA concentration and fatty acid content were determined by gas chromatog-

raphy. Two milliliters of fermentation fluid was centrifuged at 150,000×g for 10 min, and 1 L of supernatant was injected into a gas chromatograph (Agilent 6890N GC system, USA). Peak area external standard method was used to calculate VFA and fatty acid contents. DM, NDF, and ADF contents in substrates and fermentation residues were determined according to GB6435-86, GB/T20806-2006, and NY/T1459-2007, respectively, and corresponding degradation rates were calculated based on mass relationships before and after fermentation.

1.6 Statistical Analysis

Dynamic fermentation parameters were calculated using the NON-LINEAR method in SAS 9.3 software based on the following model:

$$GP_t = B \times (1 - e^{-ct})$$

Where: GP is gas production from 0.5 g substrate at time t (mL); B is theoretical maximum gas production from 0.5 g substrate at 24 h (mL); c is gas production rate (h^{-1}); and t is in vitro fermentation time (h).

Statistical analysis was performed using the MIXED procedure in SAS 9.3 software, with fermentation bottle as a random factor and treatment as a fixed factor. Polynomial contrasts were used to test linear and quadratic effects of treatments. Results are presented as least squares means. Significance was declared at $P<0.05$, and trends were identified at $0.05 \leq P<0.10$.

Results

2.1 Effects of Scleropyrum wallichianum Oil on 24 h Gas Production Parameters

The effects of different dietary supplementation levels of *Scleropyrum wallichianum* oil on 24 h in vitro rumen gas production parameters are presented in Table 3 . Gas production, theoretical maximum gas production, and gas production rate were not affected by oil supplementation ($P>0.05$) and showed no linear or quadratic trends ($P>0.05$). The 1%, 2%, and 3% groups exhibited a trend ($0.05 \leq P<0.10$) for higher gas production and theoretical maximum gas production compared to the control and 4% groups.

2.2 Effects of Scleropyrum wallichianum Oil on 24 h Fermentation Parameters

The effects of different dietary supplementation levels of *Scleropyrum wallichianum* oil on 24 h in vitro rumen fermentation parameters are shown in Table 4 . Fermentation fluid pH was not affected by oil supplementation ($P>0.05$). NH_3-N concentration increased quadratically with increasing oil supplementation ($P=0.044$). TVFA ($P<0.001$), acetate ($P<0.001$), propionate ($P=0.047$), and

butyrate concentrations (P=0.017) all increased quadratically with increasing supplementation. TVFA, acetate, and propionate concentrations in all treatment groups were significantly higher than in the control group (P<0.05), and TVFA and acetate concentrations in the 1%, 2%, and 3% groups were significantly higher than in the 4% group. MCP concentration, DMD, NDFD, and ADFD were not affected by oil supplementation (P>0.05).

2.3 Effects of Scleropyrum wallichianum Oil on 24 h Fatty Acid Composition

The effects of different dietary supplementation levels of *Scleropyrum wallichianum* oil on 24 h in vitro rumen fatty acid composition are presented in Table 5 . The contents of medium- and long-chain saturated fatty acids C12:0–C17:0 and long-chain saturated fatty acids including C20:0, C22:0, and C24:0 were not affected by oil supplementation (P>0.05). Saturated fatty acid content decreased linearly (P=0.008) and quadratically (P=0.028) with increasing oil supplementation, with the 3% and 4% groups being significantly lower than the control and 1% and 2% groups (P<0.05). Long-chain saturated fatty acid C18:0 content decreased linearly (P=0.030), with the 3% group significantly lower than all other groups (P<0.05). Unsaturated fatty acid content increased linearly (P=0.008) and quadratically (P=0.028) with increasing supplementation, with the 3% group significantly higher than all other groups (P<0.05). Specifically, t9-C18:1 content increased linearly (P=0.002), t11-C18:1 content increased quadratically (P<0.001), and c9-C18:1 content increased both linearly (P=0.028) and quadratically (P=0.005), with t9-C18:1 and c9-C18:1 reaching maximum values at the 3% supplementation level. Polyunsaturated fatty acid C18:2 content was not affected by oil supplementation (P>0.05).

Discussion

3.1 Effects of Scleropyrum wallichianum Oil on 24 h Gas Production Parameters

In this study, supplementation with different levels of *Scleropyrum wallichianum* oil had no significant effects on 24 h gas production, theoretical maximum gas production, or gas production rate. When supplementation levels were 1%, 2%, and 3%, 24 h gas production and theoretical maximum gas production numerically increased and were higher than the control group. However, when supplementation increased to 4%, these parameters showed a decreasing trend and fell below control values. Gas production serves as a comprehensive indicator reflecting dietary degradability, representing both overall rumen microbial activity and feed digestibility. Stronger microbial activity and higher dietary degradability result in greater gas production. In this study, the changes in gas production correlated with changes in DMD, suggesting that this trend may be related to the effects of *Scleropyrum wallichianum* oil supplementation on rumen microbial activity.

3.2 Effects of Scleropyrum wallichianum Oil on 24 h Fermentation Parameters

Rumen fluid pH is an important indicator reflecting the degree and pattern of rumen fermentation [13], influenced by factors such as saliva secretion and VFA composition [14]. Under normal conditions, rumen fluid pH ranges from 6.0 to 7.0. In this study, pH values across all supplementation levels remained within the normal range without significant differences, indicating that the tested *Scleropyrum wallichianum* oil levels did not cause pH imbalance in the fermentation fluid.

Rumen fluid NH_3-N concentration is a crucial indicator of rumen nitrogen metabolism, primarily affected by the combined influence of rumen microbial degradation of dietary crude protein and MCP synthesis [15], with an optimal range of 8.5–30.0 mg/dL. In this study, NH_3-N concentrations in both control and treatment groups exceeded this range, likely because the in vitro culture system cannot truly simulate in vivo rumen fermentation and lacks absorption and excretion functions, resulting in direct dissolution of produced NH_3-N into the fermentation fluid and consequently higher concentrations [16]. The quadratic change in NH_3-N concentration with increasing oil supplementation may be attributed to supplemental fat providing energy for rumen bacteria, enhancing their activity [17] and improving degradation capacity for crude protein and other components. However, White [18] and Ørskov et al. [19] reported that excessive dietary fat inhibits rumen microbial metabolic activity, with this inhibitory effect positively correlated with fatty acid unsaturation and concentration [17]. *Scleropyrum wallichianum* oil contains high levels of C18:2 (53.42%), which may have inhibited rumen microorganisms at the 4% supplementation level. These factors likely contributed to the observed quadratic trend of NH_3-N concentration increasing initially then decreasing with higher supplementation levels. The lack of significant change in MCP concentration may be because MCP synthesis was already saturated at the existing NH_3-N concentration levels.

VFA are the primary products of carbohydrate fermentation in the rumen and the main energy source for ruminants, with their concentration and composition reflecting rumen digestive metabolism [4]. The effects of dietary plant oils on VFA production are complex and depend on oil type and supplementation level. Clinquart et al. [20] reported that fat supplementation below 3.3% (DM basis) increased VFA concentration, while levels above 5.0% (DM basis) decreased it. In this study, VFA concentrations increased when *Scleropyrum wallichianum* oil supplementation was below 3% and showed a decreasing trend at 4% supplementation, consistent with previous reports. Some studies have shown that dietary plant oils decrease acetate and butyrate concentrations while increasing propionate, thereby reducing the acetate-to-propionate ratio. Ueda et al. [21] added 3% linseed oil (DM basis) to diets with different concentrate-to-forage ratios and observed significantly decreased acetate and butyrate concentrations with increased propionate. Jenkins [22] reported that 10% soybean oil supplementa-

tion in wethers' diets decreased acetate and increased propionate. In this study, propionate concentration increased significantly, consistent with these reports. This increase is generally attributed to supplemental fat being hydrolyzed by lipase into long-chain fatty acids and glycerol, with glycerol subsequently converting to propionate [23]. The changes in acetate and butyrate in this study differed from previous reports, possibly due to variations in unsaturated fatty acid content among different plant oils.

3.3 Effects of Scleropyrum wallichianum Oil on 24 h Fatty Acid Composition

The rumen is a highly reduced environment where dietary fats are degraded by rumen microorganisms into glycerol and fatty acids, while unsaturated fatty acids (C18:1, C18:2, and C18:3) undergo biohydrogenation, generating intermediate products such as C18:1 and conjugated linoleic acid (CLA) [24]. Wu et al. [25] reported that supplementing diets with 5% and 10% linseed oil (substrate DM basis) increased CLA and other unsaturated fatty acids in fermentation fluid. Gunal et al. [24] found that adding eucalyptus oil at 125, 250, and 500 mg/L to fermentation fluid increased unsaturated fatty acid content while decreasing the hydrogenation product C18:0.

In this study, all monounsaturated fatty acids (c9-C18:1, t11-C18:1, and t9-C18:1) increased quadratically with increasing *Scleropyrum wallichianum* oil supplementation, likely because the oil' s high unsaturated fatty acid content generated these intermediates during biohydrogenation. Yang [17] investigated the effects of 4% soybean oil and linseed oil supplementation in dairy cow diets on rumen microbial hydrogenating bacteria using real-time PCR, finding that plant oil supplementation significantly reduced the populations of *Butyrivibrio fibrisolvens* and *Ruminococcus albus*. In this study, c9-C18:1 and t9-C18:1 contents reached maximum values at the 3% supplementation level and decreased at 4%, possibly because excessive fat reduced hydrogenating bacterial populations, decreasing the degree of unsaturated fatty acid hydrogenation and consequently reducing intermediate products c9-C18:1, t11-C18:1, and t9-C18:1. The linear decrease in C18:0 content with increasing supplementation may be due to high dietary C18:2 inhibiting the hydrogenation of t11-C18:1 to C18:0 [26].

Conclusions

Supplementation with appropriate levels of *Scleropyrum wallichianum* oil increased NH_3-N, TVFA, acetate, propionate, and butyrate concentrations, as well as unsaturated fatty acids c9-C18:1, t11-C18:1, and t9-C18:1 content, while decreasing saturated fatty acid C18:0 content in 24 h in vitro rumen fermentation fluid. Under the conditions of this experiment, the appropriate supplementation level of *Scleropyrum wallichianum* oil is 3%.

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