Effects of Different Combinations of Long-Chain Fatty Acids on In Vitro Rumen Bacterial Fermentation and Community Structure[1]Postprint

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Abstract

This study aimed to investigate the effects of different long-chain fatty acid combinations on in vitro rumen bacterial fermentation and community structure. Rumen fluid was obtained from three rumen-fistulated dairy cows. The control group (A) substrate contained 5% calcium fatty acid, while experimental group substrates contained stearic acid, oleic acid, linoleic acid, and linolenic acid at concentrations of 1.5%, 1.0%, 0.5%, and 1.5% (group B), 1.5%, 1.0%, 1.5%, and 1.0% (group C), 1.0%, 1.5%, 1.5%, and 0.5% (group D), and 1.5%, 0.5%, 0.5%, and 1.0% (group E), respectively. Culture fluid was collected at 0, 3, 6, 12, 18, and 24 h of incubation to determine pH, ammonia nitrogen concentration, and rumen bacterial abundance. The results showed: 1) Culture fluid pH did not differ significantly among groups ($P>0.05$); ammonia nitrogen concentration in group C was significantly higher than that in groups B and D ($P<0.05$). 2) Except for *Ruminococcus albus*, the abundances of other bacterial genera differed significantly among groups ($P<0.05$). Specifically, *Bacteroides succinogenes*, *Ruminococcus flavefaciens*, *Clostridium proteoclasticum*, and *Ruminobacter amylophilus* exhibited higher abundances in group B; *Butyrivibrio fibrisolvens*, *Megasphaera elsdenii*, *Ruminococcus amylophilus*, and total rumen bacterial abundance in group C were significantly higher than those in other groups ($P<0.05$). *Megasphaera elsdenii* was the most abundant bacterium in the culture fluid, constituting the dominant species. In conclusion, fatty acid combinations significantly influenced total rumen bacteria and most bacterial species, which was associated with the fermentation pattern.

Full Text

Preamble

Effects of Long-Chain Fatty Acid Combinations on Ruminal Bacterial Fermentation and Community Structure In Vitro

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Abstract: This experiment was conducted to investigate the effects of different long-chain fatty acid combinations on ruminal bacterial fermentation and community structure in vitro. Rumen fluid was obtained from three dairy cows fitted with ruminal fistulas. The substrate of the control (A) group contained 5% fatty acid calcium, while the experimental groups contained varying proportions of stearic acid, oleic acid, linoleic acid, and linolenic acid: 1.5%, 1.0%, 0.5%, and 1.5% (B group); 1.5%, 1.0%, 1.5%, and 1.0% (C group); 1.0%, 1.5%, 1.5%, and 0.5% (D group); and 1.5%, 0.5%, 0.5%, and 1.0% (E group). Culture medium was collected at 0, 3, 6, 12, 18, and 24 h post-incubation for determination of pH, ammonia nitrogen concentration, and ruminal bacterial content. The results showed: 1) No significant differences were observed in culture medium pH among groups ($P>0.05$), while ammonia nitrogen concentration in group C was significantly higher than that in groups B and D ($P<0.05$). 2) Except for *Ruminococcus albus*, significant differences were found in the abundance of other bacterial genera among groups ($P<0.05$). *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Clostridium proteoclasticum*, and *Ruminobacter amylophilus* were more abundant in group B, whereas group C exhibited significantly higher levels of *Butyrivibrio fibrisolvens*, *Megasphaera elsdenii*, *Ruminococcus bromii*, and total bacteria compared to other groups ($P<0.05$). *Megasphaera elsdenii* was the dominant genus with the highest abundance. In conclusion, fatty acid combinations significantly affected the abundance of total ruminal bacteria and most bacterial taxa, which was related to fermentation patterns.

Keywords: fatty acid combination; rumen; bacteria; community structure

Rumen microorganisms in ruminants are primarily composed of bacteria, protozoa, archaea, and fungi, with their population size and structure closely related to host health, feed utilization efficiency, and production performance. Lipid supplementation can influence ruminal microbial populations and structure by regulating the abundance of various microbial groups, protozoal predation on bacteria, or interactions with archaea, thereby affecting rumen fermentation and ultimately host feed utilization and performance. Previous studies have reported that adding 5% and 10% linseed oil to *Leymus chinensis* substrate significantly reduced gas and methane production while increasing hydrogen output during ruminal microbial fermentation. Furthermore, in vitro cultivation with plant oils of different saturation levels affected enzyme activity, microbial vital-

ity, ruminal protozoa, bacterial protein, and DNA. Research on fatty acids has demonstrated that different ratios of linoleic to linolenic acid influence artificial rumen fermentation and methane production, with effects intensifying as the proportion of linolenic acid increases. Different fatty acids also exert varying effects on acetate and propionate production during ruminal fermentation. Our previous study on six different long-chain fatty acids indicated that saturation degree could modulate ruminal microbial fermentation patterns, and orthogonal testing identified optimal fatty acid combinations for specific fermentation modes (acetate-type, propionate-type, butyrate-type, etc.). However, whether these combinations influence fermentation patterns by affecting ruminal bacterial community structure remains unclear. Therefore, this study employed the selected fatty acid combinations for in vitro ruminal microbial cultivation to examine the abundance of different bacterial taxa, aiming to provide fundamental data for elucidating the mechanisms by which fatty acids affect ruminal microecology in ruminants.

1.1 Experimental Animals and Management

Three Holstein dairy cows aged 4 years, weighing 560$\pm$18$kgwithanaveragemilkyieldof15.5\pm$0.5 kg and fitted with permanent ruminal fistulas, were selected from the dairy farm at Yangzhou University Experimental Agriculture and Animal Husbandry Station to provide rumen fluid. During the experimental period, the fistulated cows were fed a conventional diet (concentrate-to-forage ratio of 40:60) provided by the dairy farm, with equal portions at 07:00 and 19:00 daily, and free access to water.

1.2 Experimental Design and Culture Substrates

Based on our previous research using in vitro simulated ruminal fermentation technology to cultivate ruminal microorganisms with four representative fatty acids (stearic acid, oleic acid, linoleic acid, and α-linolenic acid), optimal combinations for acetate, propionate, butyrate, and total volatile fatty acid fermentation were obtained. According to these optimal ratios, five groups were designed: Group A (control) contained 5% calcium palmitate in the culture substrate; groups B, C, D, and E contained different combinations of stearic acid, oleic acid, linoleic acid, and linolenic acid: B (1.5%, 1.0%, 0.5%, and 1.5%, acetate-type fermentation), C (1.5%, 1.0%, 1.5%, and 1.0%, propionate-type fermentation), D (1.0%, 1.5%, 1.5%, and 0.5%, butyrate-type fermentation), and E (1.5%, 0.5%, 0.5%, and 1.0%, total volatile fatty acid-type fermentation). The deficit to reach 5% was supplemented with calcium palmitate. Each group had three replicates, with one blank control without substrate. The composition of culture substrates is shown in Table 1 .

1.3 In Vitro Cultivation

In vitro cultivation followed the method of Menke et al. Rumen fluid was collected from three fistulated cows before morning feeding. Culture medium was

prepared by mixing artificial saliva salts and rumen fluid at a 2:1 ratio, pre-warmed at 39°C under CO_2. Exactly 1.50 g of substrate from each group was weighed into culture bottles, and 150 mL of culture medium was added to each bottle. The bottles were incubated at 39°C with shaking at 50 r/min under CO_2. Culture medium was collected at 0, 3, 6, 12, 18, and 24 h post-incubation for subsequent analysis.

1.4 Primer Design

The 16S rDNA sequences of different strains, including *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, were retrieved from GenBank. Intraspecific conserved regions were identified through sequence alignment using MegAlign in DNAStar. Primers were designed using Primer Express 5.0 and specificity was verified by BLAST in GenBank. Primer sequences are listed in Table 2 .

Table 2. PCR Primers Sequence of Main Microbes

Items	Primers Sequence (5' -3')	Fragments/bp
Fibrobacter succinogenes	F: GTT CGG AAT TAC TGG GCG TAA AR: CGC CTG CCC CTG AAC TAT C	
Ruminococcus flavefaciens	F: ATT GTC CCA GTT CAG ATT GCR: GGC GTC CTC ATT GCT GTT AG	
Ruminococcus albus	F: TCT GTC TTT GGG GAC GAT AAR: AAG TGC AGT TCA GGG TTA A	
Butyrivibrio fibrisolvens	F: TAA CAT GAG AGT TTG ATC CTG GCT CR: CGT TAC TCA CCC GTC CGC	
Megasphaera elsdenii	F: GAT TCT GGC TCA GGA TGA ACGR: CGG GTG CTI CCC ACT TTC ATG	
Clostridium proteoclasticum	F: TCC GGT GGT ATG AGA TGG GCR: GTC GCT GCA TCA GAG TTT CCT	
Ruminobacter amylophilus	F: TGA CCG CCT GGG GAG TAC GGR: TTG CGC TCG TTG CGG GAC TT	
Ruminococcus bromii	F: TTT GTC AAC GGC AGT CCT ATTR: AC CAG GTC TTG ACA TCG AGT G	
Bacterial-U	F: GAG GCA GCA GTA GGG AAR: CAG CGT CAG TTA CAG ACC AGA G	

1.5 Index Determination

Sample pH was measured immediately using a Shanghai Leici pHS-3C pH meter. Ammonia nitrogen concentration was determined using the method of Feng Zongci et al. Ruminal bacterial DNA was extracted using the bead-beating method of Zoetendal et al. Quantitative detection was performed by real-time quantitative PCR (RT-PCR) on a 7500 RT-PCR instrument, with Bacterial-U as the internal reference.

1.6 Microbial Content Calculation

The relative quantification method was used to quantify each bacterium, expressed as the percentage of each bacterium relative to total ruminal bacterial 16S rDNA. The calculation formula was:

Target bacteria content (%) = 2^-(Ct_{target} - Ct_{total} bacteria)

Where: Ct_{target} is the cycle threshold of the target bacterium, and Ct_{total} bacteria is the cycle threshold of total bacteria.

1.7 Statistical Analysis

Data were compiled using Excel 2003 and analyzed using the General Linear Model Univariate procedure in SPSS 17.0 software. Differences were considered significant at $P<0.05$.

2.1 Effects of Different Fatty Acid Combinations on Culture Medium pH

As shown in Table 3 , culture medium pH ranged from 6.02 to 6.52. The effect of fatty acid combinations was not significant ($P>0.05$), while the effect of time was significant ($P<0.05$), with the highest pH observed at 3 h post-incubation. A significant interaction between time and fatty acid combination was observed ($P<0.05$): pH increased from 0 to 3 h and decreased from 3 to 12 h in all groups with similar trends; however, from 12 to 18 h, all groups except A decreased, while from 18 to 24 h, groups A and B decreased whereas groups C, D, and E increased.

2.2 Effects of Different Fatty Acid Combinations on Ammonia Nitrogen Concentration

Table 4 shows that ammonia nitrogen concentration ranged from 7.91 to 17.05 mg/dL. The effect of fatty acid combinations was significant ($P<0.05$), with group C showing the highest concentration. The effect of time was also significant ($P<0.05$), with lower concentrations at 0 and 3 h. A significant interaction between time and fatty acid combination was observed ($P<0.05$): from 0 to 3 h, all groups except A increased; from 3 to 6 h, all groups except D increased; from 6 to 12 h, all groups except B decreased; from 12 to 18 h, all groups except

C increased; and from 18 to 24 h, groups A and E decreased while groups B, C, and D increased.

2.3 Effects of Different Fatty Acid Combinations on Ruminal Bacterial Content

Table 5 shows that except for *Ruminococcus albus*, significant differences were observed in the abundance of other bacterial taxa among groups (P<0.05). *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Clostridium proteoclasticum*, and *Ruminobacter amylophilus* were more abundant in group B. *Butyrivibrio fibrisolvens* and *Ruminococcus bromii* were more abundant in group C, significantly higher than other groups (P<0.05). *Megasphaera elsdenii* was lowest in group A, significantly lower than in group C which showed the highest abundance (P<0.05).

Significant differences existed among the measured bacterial taxa (P<0.05). *Megasphaera elsdenii* was the dominant genus with the highest abundance (8.45%), while *Ruminococcus albus* (0.27%) and *Ruminococcus flavefaciens* (0.44%) were relatively low. Based on the measured taxa, total ruminal bacterial content was highest in group C, significantly higher than other groups (P<0.05).

Table 5. Effects of Different Fatty Acid Combinations on Ruminal Bacteria Contents in Culture

Items	Groups	P-value
	A	B
Fibrobacter succinogenes	1.69a	2.07a
Ruminococcus flavefaciens	0.50b	0.84a
Ruminococcus albus	0.27b	
Butyrivibrio fibrisolvens	1.05c	2.33b
Megasphaera elsdenii	1.14b	7.07ab
Clostridium proteoclasticum	1.84ab	2.37a
Ruminobacter amylophilus	1.05bc	1.90a
Ruminococcus bromii	0.65b	4.59b
Total bacteria	8.53c	21.27b

Values of different groups with different letter superscripts differed significantly (P<0.05), and means with different letter superscripts differed significantly (P<0.05).

3.1 Effects of Different Fatty Acid Combinations on Ruminal Bacterial Fermentation In Vitro

In this experiment, culture medium pH ranged from 6.02 to 6.52, which was suitable for ruminal microbial growth. This result is consistent with previous

studies on oils or fatty acids. Song Zhigang et al. reported that adding 4% of four plant oils with different saturation levels did not significantly affect pH in vitro. Pi Yu et al. also found no significant pH differences when adding 3% of six fatty acids with different saturation levels. Ammonia nitrogen concentration in this study ranged from 7.91 to 17.05 mg/dL, which is within the suitable range for ruminal microbial fermentation and growth. However, ammonia nitrogen concentration differed significantly among fatty acid combination groups, with group C being highest and group D lowest (C > E > B > D). The unsaturation degrees of fatty acid combinations in groups B, C, D, and E were 0.92, 1.00, 0.50, and 0.71, respectively (C > B > E > D), showing that ammonia nitrogen concentration generally corresponded to unsaturation degree. One possible reason is that as unsaturation degree decreases, the inhibitory effect on protozoal populations and their predatory activity on bacteria increases, leading to higher bacterial numbers and enhanced fermentation activity, which may result in elevated ammonia nitrogen concentration. This is consistent with the ranking of total bacterial content (C > B > E > D). These results suggest that different fatty acid combinations affect fermentation by influencing ruminal bacterial communities. Temporal analysis revealed that the higher average ammonia nitrogen concentration in group C was mainly due to enhanced bacterial fermentation activity during 3–12 h, while increased bacterial growth and ammonia utilization during 18–24 h led to lower ammonia nitrogen concentration compared to group D.

3.2 Effects of Different Fatty Acid Combinations on Ruminal Bacteria In Vitro

Most previous reports have focused on the effects of specific oils or fatty acids on ruminal bacteria, with few systematic studies on multiple fatty acids and their combined effects. This study further explored the effects of optimal fatty acid combinations for different fermentation modes identified in our previous research. Group C showed the highest total bacterial content, which may be related to its higher unsaturation degree and potential synergistic effects among fatty acids. Concurrently, group C also had the highest *Butyrivibrio fibrisolvens* abundance. This may be attributed to several factors: first, *B. fibrisolvens* primarily resides in the liquid phase, and protozoa predominantly ingest liquid-phase bacilli and cocci (0.2–1.0 m), while the unsaturated bonds in this group inhibited protozoal predation; second, the addition of appropriate amounts of unsaturated oil (linoleic acid) may have promoted bacterial proliferation and its hydrogenation function. Lee et al. reported that fish oil supplementation increased linoleic acid biohydrogenation. *B. fibrisolvens* metabolizes diverse substrates in the rumen, with butyrate and lactate as main fermentation products, and interacts with *Megasphaera elsdenii* and *Selenomonas ruminantium* to maintain ruminal pH stability. The lactate produced can be utilized by *M. elsdenii* to generate propionate via the acrylate-CoA pathway. The highest *M. elsdenii* abundance in group C may explain why this fatty acid combination produced the highest propionate content.

Ruminococcus bromii also primarily resides in the ruminal liquid phase and mainly metabolizes starch, being relatively active under high-concentrate, low-pH conditions. Studies on dietary soybean and linseed oil supplementation in beef cattle did not find changes in ruminal starch-degrading bacteria, but this study found the highest *R. bromii* abundance in group C. This may be due to reduced protozoal predation on liquid-phase bacteria resulting from higher unsaturation degree. The increased abundance of this bacterium and its interactions with other taxa may also contribute to increased propionate production from starch metabolism, suggesting that the propionate-type fermentation fatty acid combination may function by elevating *R. bromii* abundance.

Ruminobacter amylophilus is another ruminal starch-degrading bacterium that primarily produces acetate and succinate. Its highest abundance in group B may partially explain why group B was the acetate-type fermentation combination. Additionally, *R. amylophilus* is a major proteolytic bacterium that degrades protein into ammonia nitrogen and amino acids for growth. The continuously increasing ammonia nitrogen concentration in group B aligns with its higher *R. amylophilus* abundance, consistent with Wang Mengzhi et al.' s finding of higher abundance of this bacterium in ruminal fluid with high ammonia nitrogen concentration from soybean meal diets.

Cellulose-degrading bacteria such as *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes* primarily reside in the ruminal solid phase, producing acetate as the main fermentation product. High-fiber diets have been shown to increase *R. flavefaciens* and *F. succinogenes* populations in cattle. In this study, both *R. flavefaciens* and *F. succinogenes* were more abundant in group B. The increased *R. flavefaciens* in group B is consistent with Jin Long et al.' s finding that 4% palm kernel oil increased this bacterium in vitro, while the increased *F. succinogenes* differs from Wurina' s result that 6% plant oil significantly reduced this bacterium. This discrepancy may be due to oil addition exceeding certain levels, affecting bacterial activity and enzyme secretion by adhering to cellulose and bacterial surfaces or damaging cell membranes of some cellulose-degrading microbes. The increased abundance of these two taxa in group B, consistent with the higher *R. amylophilus* abundance, suggests that group B' s fatty acid combination may enhance acetate fermentation by altering ruminal bacterial composition. However, *R. albus* abundance did not significantly increase in group B, consistent with Jin Long et al.' s finding that 4% coconut or palm kernel oil did not affect *R. albus* abundance. Some studies suggest that *R. albus* produces antibiotics that inhibit *R. flavefaciens* growth. In this study, the ranking of *R. albus* abundance was D > C > E > B, while *R. flavefaciens* abundance was B > E > C > D, revealing an inverse relationship that suggests potential mutual inhibition, though the underlying mechanism requires further investigation.

Conclusions

1. Supplementation with different fatty acid combinations resulted in culture medium pH and ammonia nitrogen concentrations within suitable ranges, but significantly altered total ruminal bacterial abundance and some bacterial taxa, which was related to fermentation patterns.
2. When culture substrate contained stearic acid, oleic acid, linoleic acid, and linolenic acid at 1.5%, 1.0%, 0.5%, and 1.5%, respectively, the culture medium showed higher abundances of *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Clostridium proteoclasticum*, and *Ruminobacter amylophilus*.
3. When culture substrate contained stearic acid, oleic acid, linoleic acid, and linolenic acid at 1.5%, 1.0%, 1.5%, and 1.0%, respectively, the culture medium showed higher abundances of *Butyrivibrio fibrisolvens*, *Ruminococcus bromii*, and *Megasphaera elsdenii*.

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