Effects of different long-chain fatty acids on community structure of goat rumen protozoa and protozoal bacterial grazing cycle under in vitro culture conditions [1] Postprint

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Abstract

This study aimed to investigate the effects of different long-chain fatty acids on rumen protozoal population structure and the protozoal predation on bacteria cycle in goats under in vitro culture conditions. Six long-chain fatty acids with varying degrees of unsaturation were selected [stearic acid (Group A), oleic acid (Group B), linoleic acid (Group C), -linolenic acid (Group D), arachidonic acid (Group E), and eicosapentaenoic acid (Group F), with the number of double bonds increasing sequentially from 0 to 5], each added at 3% of the substrate level for in vitro cultivation. The predation rate of protozoa on bacteria was measured after 10 h of in vitro culture, while bacterial density, protozoal density, bacterial protein content, and protozoal protein content were determined after 24 h of in vitro culture. The results showed that, except for the proportion of Ophryoscolecinae in Group F being significantly higher than that in Groups B and D ($P<0.05$), the addition of different long-chain fatty acids had no significant effects on the proportions of Dasytricharuminantium, Entodinium, Diplodiniinae, Isotrichidae, and Epidinium in protozoa ($P>0.05$). Protozoal density was highest in Group A, which was significantly higher than that in Group B ($P<0.05$); bacterial density was highest in Group D, which was significantly higher than those in Groups A, B, E, and F ($P<0.05$). The predation rates of protozoa on bacteria in Groups A–F were 244.50, 236.51, 229.60, 189.04, 200.51, and 174.24 cells/(cell · h), respectively; additionally, Group D had the lowest bacterial turnover rate (0.68%) and the longest bacterial turnover time (146.92 h). The estimated bacterial protein recycling amount was relatively low in Group D [126.75 mg/(d · head)] and Group F [131.63 mg/(d · head)]. Therefore, based on the results of this study, it is concluded that -linolenic acid exhibits a superior inhibitory effect on protozoal predation on bacteria in the goat rumen under in vitro culture conditions.

Full Text

Effects of Different Long-Chain Fatty Acids on Protozoal Community Structure and Bacterial Recycling Due to Protozoa Engulfment in Goat Rumen *in vitro*

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Abstract

This experiment was conducted to investigate the effects of different long-chain fatty acids on protozoal community structure and bacterial recycling due to protozoa engulfment in goat rumen *in vitro*. Six long-chain fatty acids with varying degrees of unsaturation were selected: stearic acid (Group A), oleic acid (Group B), linoleic acid (Group C), -linolenic acid (Group D), arachidonic acid (Group E), and eicosapentaenoic acid (Group F), with the number of unsaturated bonds increasing sequentially from 0 to 5. Each fatty acid was added at 3% of the substrate level for *in vitro* culture. The protozoal engulfment rate of bacteria was measured after 10 h of *in vitro* culture, while bacterial and protozoal densities and bacterial and protozoal protein contents were determined after 24 h of culture. The results showed that, except for the proportion of *Ophryoscolecinae* in Group F being significantly higher than that in Groups B and D ($P<0.05$), the addition of different long-chain fatty acids had no significant effects on the proportions of *Dasytricha ruminantium*, *Entodinium*, *Diplodiniinae*, *Isotrichidae*, and *Epidinium* ($P>0.05$). Protozoal density was highest in Group A, which was significantly higher than that in Group B ($P<0.05$). Bacterial density was highest in Group D, which was significantly higher than that in Groups A, B, E, and F ($P<0.05$). The bacterial engulfment rates of protozoa in Groups A through F were 244.50, 236.51, 229.60, 189.04, 200.51, and 174.24 cells/(cell·h), respectively. Additionally, Group D exhibited the lowest bacterial turnover rate (0.68%) and the longest bacterial turnover time (146.92 h). The estimated bacterial protein recycling amounts were relatively low in Groups D [126.75 mg/(d·head)] and F [131.63 mg/(d·head)]. Based on these results, it is concluded that -linolenic acid exhibits the best inhibitory effect on bacterial recycling due to protozoa engulfment in goat rumen under *in vitro* culture conditions.

Keywords: fatty acids; unsaturated bonds; protozoa; engulfing bacteria

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Introduction

Rumen microbial protein (MCP) is one of the main nitrogen sources for ruminants, providing 40%-80% of their protein requirements. Its synthesis level can reflect the microbial population and the efficiency of microbial protein synthesis in the rumen and is related to the effective utilization of nitrogen sources by the

rumen. The nitrogen cycle in the rumen caused by protozoal engulfment and digestion of bacteria undoubtedly reduces nitrogen utilization efficiency, making protozoa one of the primary reasons for low nitrogen metabolism efficiency in the rumen. Studies have shown that defaunation facilitates the transfer of urea nitrogen to the rumen, reduces ruminal nitrogen cycling, and promotes bacterial protein synthesis. Oils and fatty acids, as excellent energy supplements for ruminants, have negative effects on protozoal growth and can promote rumen microbial protein synthesis while reducing ruminal nitrogen cycling. Reports indicate that plant oils rich in C18 fatty acids can reduce methane production and improve the rumen fermentation environment. Furthermore, studies have demonstrated that adding plant oils to *in vitro* cultures affects culture fluid enzyme activity, microbial vitality, rumen protozoal numbers, and bacterial protein and DNA content. Numerous studies have clearly shown that the negative effects of unsaturated fatty acids on rumen protozoa and bacteria are related to their degree of unsaturation, but the pattern of how unsaturation affects protozoal engulfment activity and protozoal community structure remains unclear. This experiment selected six long-chain fatty acids with different degrees of unsaturation—stearic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid—with the number of unsaturated bonds increasing sequentially from 0 to 5, aiming to systematically investigate the effects of long-chain fatty acid unsaturation on rumen protozoal community structure and bacterial recycling due to protozoa engulfment, thereby providing reference data for reducing nitrogen loss during bacterial recycling by protozoa and improving nitrogen utilization efficiency in diets.

Materials and Methods

1.1 Experimental Animals and Management Three Saanen dairy goats aged 1.5 years with body weight of (29.4±2.7) kg, fitted with permanent rumen fistulas, were used. They were housed individually and fed a basal diet of corn + soybean meal + Leymus chinensis with a concentrate-to-forage ratio of 2:8. Feed was provided at 2.5% of body weight in dry matter, divided into two equal portions fed at 07:00 and 19:00 daily, with free access to water.

1.2 Experimental Design and *in vitro* Culture Substrates Six *in vitro* culture substrates were prepared, with long-chain fatty acids added as follows: stearic acid (Group A), oleic acid (Group B), linoleic acid (Group C), -linolenic acid (Group D), arachidonic acid (Group E), and eicosapentaenoic acid (Group F). The fatty acids were added at 3% of the substrate level. The composition of the *in vitro* culture substrates is shown in Table 1 . Stearic acid (Cat# 21-12-8), oleic acid (Cat# 112-80-1), linoleic acid (Cat# 60-33-3), -linolenic acid (Cat# 463-40-1), and arachidonic acid (Cat# 506-32-1) were purchased from Beijing Beilingwei Chemical Technology Co., Ltd. with purity 99.0%. Eicosapentaenoic acid (Cat# 10417-94-4) was purchased from Shanghai Kewei Chemical Technol-

ogy Co., Ltd. with purity 99.0%. Xylan (Cat# 9014-63-5), arabinan (Cat# 87-72-9), glucan (Cat# 9004-54-0), mannan (Cat# 9036-88-8), pectin (Cat# 9000-69-5), and lignin (Cat# 8061-51-6) were purchased from Sigma-Aldrich (USA) with purity 99.0%. Cellobiose (Cat# 528-50-7) and soluble starch (Cat# 9005-84-9) were purchased from Sinopharm Chemical Reagent Co., Ltd. with purity 99.0%. Urea (Cat# 57-13-6) was purchased from Beijing Huibaolianhua Technology Co., Ltd. with purity 99.0%. Casein (Cat# 9000-71-9) was purchased from Beijing Solarbio Technology Co., Ltd. with purity 99.0%.

1.3 *in vitro* Culture Procedure The *in vitro* culture procedure followed the method of Menke et al. [17] with slight modifications to substrate and culture fluid preparation. Rumen fluid was collected from the three fistulated goats before morning feeding. Culture fluid was prepared by mixing artificial saliva salts with rumen fluid at a 2:1 ratio, pre-warmed at 39°C under CO . Exactly 1.50 g of culture substrate from each group was weighed into a 250 mL Erlenmeyer flask, and 150 mL of culture fluid was added. The flasks were incubated at 39°C with shaking at 50 r/min under CO . Each group had three replicates. The protozoal engulfment experiment was conducted at 10 h of culture, and the culture was terminated at 24 h. Twenty milliliters of culture fluid was dispensed into two centrifuge tubes: one for bacterial and protozoal counting, and the other for bacterial and protozoal protein content determination.

1.4.1 Bacterial Counting Samples were stained with 2% crystal violet solution (2 g crystal violet dissolved in 10 mL 95% ethanol and 90 mL 0.8% ammonium oxalate solution) and counted using a 16×25 hemocytometer under a microscope (Olympus CKX41, Japan) at 1000× magnification. The bacterial density was calculated as follows:

$$\text{Bacterial density (cells/mL)} = \text{N/S} \times \text{D} \times 16 \times 10 \times 1000 = \text{N/S} \times \text{D} \times 16 \times 10$$

where N is the total number of counted squares, S is the number of squares counted, and D is the dilution factor.

1.4.2 Protozoal Counting Protozoal counting was performed according to the methods of Lu Dexun et al. [18] and Nsabimana et al. [19]. Samples were stained with MFS stain (8 g NaCl, 0.6 g methyl green, 100 mL formalin solution, diluted to 1000 mL) and counted using a 16×25 hemocytometer under a microscope (Olympus CKX41, Japan) at 1000× magnification. The protozoal density was calculated as follows:

$$\text{Protozoal density (cells/mL)} = \text{N}/4 \times \text{D} \times 16 \times 10 \times 1000 = \text{N} \times \text{D} \times 4 \times 10$$

where N is the total number of four medium squares counted, and D is the dilution factor.

1.4.3 Protozoal Species Identification and Counting Protozoa were identified to the genus level through morphological observation, and counting was performed as described above.

1.5 Determination of Bacterial Crude Protein and Protozoal Crude Protein Contents Culture fluid was mixed with an equal volume of physiological saline and incubated at 39°C with shaking (125 r/min) for 60 min with intermittent stirring. The mixture was then filtered through four layers of gauze, and the filtrate was centrifuged (150×g, 10 min). The precipitate (protozoa) was collected, washed twice with physiological saline, resuspended in physiological saline, and stored at -20°C for later analysis. The supernatant from the first centrifugation was further centrifuged at high speed (22,000×g, 15 min), and the precipitate (bacteria) was collected. The bacterial and protozoal samples were lyophilized, and crude protein content was determined using the Kjeldahl method to calculate bacterial and protozoal protein contents in the culture fluid.

1.6.1 Preparation of Protozoa-Free Rumen Fluid and Fluorescence-Labeled Rumen Bacteria (FLRB) Preparation of protozoa-free rumen fluid: Rumen fluid was collected and filtered through a 2 μm membrane. The filtrate was centrifuged (22,000×g, 15 min), and the precipitate was collected, washed and centrifuged twice with sterile physiological saline, then resuspended in sterile physiological saline.

Preparation of FLRB: The protozoa-free rumen fluid was stained with 1.5 mL of 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) at 60°C for 2 h, then centrifuged (22,000×g, 15 min). The supernatant DTAF solution was discarded, and the pellet was washed and centrifuged three times with sterile physiological saline, then resuspended in sterile physiological saline of the same volume as the original sample and stored at -20°C. Before use, the stored FLRB was thawed and centrifuged (22,000×g, 15 min), then resuspended in the corresponding protozoa-free rumen fluid to prepare a 2× concentrated FLRB suspension.

1.6.2 Protozoal Engulfment Experiment The method followed reference [20] with slight modifications: (1) Two sets of culture substrates were prepared according to the experimental design; one set was reserved without culture fluid, while the other was cultured *in vitro* as described above. (2) FLRB was thawed and centrifuged, then mixed with 1/2 volume of protozoa-free rumen fluid and an appropriate amount of reserved substrate, and placed in a water bath at 39°C. (3) At 10 h of *in vitro* culture, an equal volume of culture fluid was collected to isolate protozoa, which were resuspended in 1/2 volume of protozoa-free rumen fluid. (4) The protozoal and labeled bacterial suspensions were combined for the engulfment experiment. (5) Samples were taken every 5 min to prepare slides, with five consecutive samplings. The number of FLRB inside protozoa was detected using an inverted fluorescence microscope (Olympus CKX41, Japan).

Each group had three replicates, and each slide was counted as the average of 12 protozoa.

1.6.3 Calculation of Nitrogen Cycling Indices Between Rumen Bacteria and Protozoa Based on the protozoal engulfment rate of bacteria and protozoal density, the bacterial engulfment quantity was obtained, and combined with bacterial density, the bacterial turnover time and turnover rate caused by protozoal engulfment were calculated as follows:

Bacterial engulfment quantity [cells/(mL · h)] = Engulfment rate × Protozoal density
Bacterial turnover time (h) = Bacterial density / Bacterial engulfment quantity
Bacterial turnover rate (%) = Bacterial engulfment quantity / Bacterial density

According to reports by Sherr et al. [21], Li Hongbo et al. [22], and Wang Jialing et al. [23], the average volume of fluorescence-labeled bacteria (FLB) is 0.10 μm³, and the conversion coefficient to nitrogen is 0.054 pg/μm³. Therefore, the bacterial nitrogen engulfment rate by protozoa in the rumen was calculated as follows:

Bacterial nitrogen engulfment rate [pg/(cell · h)] = Protozoal engulfment rate × 0.054

Based on the bacterial nitrogen engulfment rate and protozoal density, the daily nitrogen recycling amount in the rumen of each goat (assuming a rumen volume of 4 L) was estimated. The bacterial protein recycling amount was then calculated from the nitrogen recycling amount:

Nitrogen recycling amount [mg/(d · head)] = Nitrogen engulfment rate × Protozoal density × 4 × 24 × 10
Bacterial protein recycling amount [mg/(d · head)] = Nitrogen recycling amount × 6.25

1.7 Statistical Analysis SPSS 13.0 software was used for regression analysis via the Regression-Curve Estimation procedure and for one-way ANOVA followed by Tukey' s multiple comparison test. $P<0.05$ was considered statistically significant.

Results

2.1 Effects of Different Long-Chain Fatty Acids on Protozoal and Bacterial Protein Contents As shown in Table 2 , protozoal protein content was highest in Group A (0.591 mg/mL), which was significantly higher than that in Groups C, D, and E ($P<0.05$), but not significantly different from Groups B and F ($P>0.05$). The ranking of protozoal protein content was Group A > Group B > Group F > Group E > Group D > Group C. Bacterial protein content was lowest in Group A (0.123 mg/mL) and highest in Group D (0.285

mg/mL), with Group D being significantly higher than Groups A, B, E, and F ($P<0.05$), but not significantly different from Group C ($P>0.05$).

2.2 Effects of Different Long-Chain Fatty Acids on Protozoal and Bacterial Densities As shown in Table 2, protozoal density was highest in Group A (2.691×10 cells/mL) and lowest in Group B (1.714×10 cells/mL), with Group A being significantly higher than Group B ($P<0.05$). No significant differences were observed among Groups A, C, D, E, and F ($P>0.05$). Bacterial density was highest in Group D (5.752×10 cells/mL) and lowest in Group A (4.728×10 cells/mL), with Group D being significantly higher than Groups A, B, E, and F ($P<0.05$), but not significantly different from Group C ($P>0.05$).

2.3 Effects of Different Long-Chain Fatty Acids on Protozoal Genus Proportions As shown in Table 3 , the proportion of *Ophryoscolecinae* was highest in Group F (5.318%), which was significantly higher than that in Groups B and D ($P<0.05$). *Ophryoscolecinae* was also the least abundant genus among the identified protozoa. The addition of different long-chain fatty acids had no significant effects on the proportions of *Dasytricha ruminantium*, *Entodinium*, *Diplodiniinae*, *Isotrichidae*, and *Epidinium* ($P>0.05$). *Dasytricha ruminantium* was highest in Group C (13.836%) and lowest in Group A (10.734%). *Entodinium* was highest in Group C (32.536%) and lowest in Group B (30.825%). *Diplodiniinae* was highest in Group E (14.380%) and lowest in Group F (10.765%). *Isotrichidae* was highest in Group A (18.091%) and lowest in Group F (14.875%). *Epidinium* was highest in Group A (15.394%) and lowest in Group C (10.724%).

2.4 Effects of Different Long-Chain Fatty Acids on Protozoal Engulfment Rate of Bacteria As shown in Figure 1 [Figure 1: see original paper], the number of bacteria engulfed by protozoa increased gradually with time, with similar overall trends across all groups. The increase was relatively rapid within 0–15 min, but the growth rate gradually plateaued after 35 min. Therefore, the data from 0–35 min were considered reliable for linear regression analysis to calculate the protozoal engulfment rate of bacteria.

Linear regression analysis between the engulfment quantity of FLRB by protozoa (cells/cell) and engulfment time (min) yielded the following regression equations for each group: Group A, $Y=14.192+3.838X$ ($R^2=0.98$; $P<0.001$; $n=9$); Group B, $Y=6.774+3.829X$ ($R^2=0.99$; $P<0.001$; $n=9$); Group C, $Y=2.202+3.790X$ ($R^2=0.99$; $P<0.001$; $n=9$); Group D, $Y=0.522+3.142X$ ($R^2=0.99$; $P<0.001$; $n=9$); Group E, $Y=2.333+3.303X$ ($R^2=0.99$; $P<0.001$; $n=9$); Group F, $Y=-0.597+2.914X$ ($R^2=0.99$; $P<0.001$; $n=9$). All equations had R^2 values above 0.98, indicating good linear relationships and reliable results.

The calculated protozoal engulfment rates of bacteria (Table 3) were 244.50, 236.51, 229.60, 189.04, 200.51, and 174.24 cells/(cell · h) for Groups A through

F, respectively. Group A (stearic acid) showed the highest rate, while Group F (eicosapentaenoic acid) showed the lowest, generally decreasing with increasing number of unsaturated bonds, although arachidonic acid was higher than linolenic acid. The bacterial nitrogen engulfment rates, calculated using conversion factors, were 1.32, 1.28, 1.24, 1.02, 1.08, and 0.94 pg/(cell · h) for Groups A through F, respectively.

2.5 Effects of Different Long-Chain Fatty Acids on Nitrogen Cycling Between Bacteria and Protozoa Based on protozoal density and engulfment rate, the bacterial engulfment quantity was calculated (Table 4). Group A showed the highest value (657.95×10 cells/(mL · h)), while Group D showed the lowest (391.50×10 cells/(mL · h)). Bacterial turnover time and turnover rate due to protozoal engulfment were calculated from bacterial density and engulfment quantity (Table 4). Group D had the lowest bacterial turnover rate (0.68%) and the longest turnover time (146.92 h), meaning approximately 0.68% of bacteria were engulfed by protozoa per hour, and bacteria would be completely turned over every 146.92 h. Group A had the highest bacterial turnover rate (1.39%) and the shortest turnover time (71.86 h). The ranking of bacterial turnover rate was Group D < Group B < Group F < Group E < Group C < Group A. The bacterial nitrogen engulfment rate (Table 4) was converted from the protozoal engulfment rate. Based on this, the daily nitrogen recycling amount in the rumen of each goat due to protozoal engulfment was estimated (Table 4): 34.10, 21.06, 31.18, 20.28, 25.67, and 23.63 mg/(d · head) for Groups A through F, respectively. The corresponding bacterial protein recycling amounts were 213.13, 131.63, 194.88, 126.75, 160.44, and 147.69 mg/(d · head), respectively.

Discussion

3.1 Effects of Different Long-Chain Fatty Acids on Rumen Protozoal Community Structure in Goat *in vitro* Protozoa can only obtain nitrogen by engulfing bacteria; therefore, their presence increases nitrogen turnover and consumption in the rumen. Studies have shown that unsaturated bonds in long-chain fatty acids can inhibit the growth of both protozoa and bacteria, leading some researchers to exploit the differential responses of protozoa and bacteria to fatty acids to suppress protozoal growth. In this experiment, Group A had the highest protozoal density, while Groups B, C, D, E, and F showed reductions of 36.31%, 2.68%, 23.04%, 7.99%, and 2.68%, respectively, compared to Group A. This indicates that unsaturated fatty acid addition inhibits rumen protozoal growth, with oleic acid and -linolenic acid being particularly effective at reducing protozoal density. Unsaturated fatty acids can decrease rumen protozoal numbers, and because of this reduction in protozoa, their engulfment of bacteria decreases. Consequently, in this experiment, bacterial density in Groups B, C, D, E, and F was higher than in Group A, likely because protozoa

are more sensitive to unsaturated fatty acids than bacteria, resulting in a greater reduction in protozoal activity than in their predation of bacteria. Therefore, bacterial density increased during the observation period, with the -linolenic acid group showing the highest bacterial density. Ivan et al. reported that C18 unsaturated fatty acids can significantly inhibit or be toxic to protozoa through hydrogen utilization in the rumen. Early studies suggested that both rumen protozoa and bacteria play roles in fatty acid hydrogenation, but later research demonstrated that protozoal hydrogenation activity mainly comes from engulfed bacteria. Additionally, the toxic effects of fatty acids on microorganisms may be related to alterations in microbial lipid bilayer cell membranes, with double bonds in fatty acids affecting the spatial structure of protozoal molecules and disrupting cell integrity.

Overall, except for *Ophryoscolecinae*, no significant differences were observed among groups for other protozoal genera. Compared with stearic acid, other long-chain fatty acids tended to reduce the proportions of *Entodinium*, *Isotrichidae*, and *Epidinium*, consistent with the protozoal density results. For *Dasytricha ruminantium*, other long-chain fatty acid groups showed an increasing trend compared to the stearic acid group, while for *Diplodiniinae*, oleic acid and arachidonic acid groups showed an increasing trend. The differential effects of long-chain fatty acid addition on different protozoal genera may be related to varying hydrogenation capacities among genera, as well as differences in reproduction rates, bacterial predation behavior, nutrient utilization, and adaptation to long-chain fatty acids. In this experiment, *Entodinium* was the dominant genus, accounting for 30%-35% of protozoa, consistent with findings by Wang Hongrong et al. and Wang Mengzhi et al. Studies have shown that *Entodinium* can selectively engulf various bacteria with a maximum engulfment capacity of up to 4,100 cells per hour. The higher proportion of *Entodinium* in the stearic acid group likely contributed to its fastest bacterial engulfment rate and highest nitrogen cycling efficiency.

3.2 Effects of Different Long-Chain Fatty Acids on Bacterial Recycling Due to Protozoa Engulfment in Goat Rumen *in vitro* The ranking of bacterial turnover rate from low to high was -linolenic acid group, oleic acid group, eicosapentaenoic acid group, arachidonic acid group, linoleic acid group, and stearic acid group. Thus, unsaturated long-chain fatty acid addition can reduce bacterial turnover rate in the rumen, with -linolenic acid being the most effective for regulating ruminal nitrogen cycling. Regarding bacterial nitrogen engulfment rate, the reduction in protozoal engulfment capacity led to slower nitrogen engulfment rates. In this experiment, the ranking of bacterial nitrogen engulfment rates was stearic acid group > oleic acid group > linoleic acid group > arachidonic acid group > -linolenic acid group > eicosapentaenoic acid group, with all groups showing reductions compared to the stearic acid group. The inability of stearic acid to reduce bacterial nitrogen engulfment rate may be because it is a saturated fatty acid with minimal or no toxic effects on rumen microorganisms, consistent with Wallace et al.' s finding that C18 fatty acids

have no significant negative effects on rumen bacteria. Oldick et al. reported that increasing the degree of fatty acid unsaturation can linearly decrease rumen protozoal numbers. Therefore, eicosapentaenoic acid showed the best effect in reducing bacterial nitrogen engulfment rate, likely due to its highest degree of unsaturation among the six fatty acids (containing five unsaturated bonds). The ranking of bacterial nitrogen engulfment rates generally corresponded to the ranking of fatty acid unsaturation from low to high, except that -linolenic acid was more effective than the more unsaturated arachidonic acid. This may be related to the position of double bonds: the first double bond in both -linolenic acid and eicosapentaenoic acid is at the third carbon atom, while in arachidonic acid it is at the sixth carbon atom.

Although protozoa have protein-degrading ability, they cannot synthesize protein, and protozoal autolysis generally provides about 20% of duodenal microbial protein. In this experiment, protozoal protein contents in the oleic acid, linoleic acid, -linolenic acid, arachidonic acid, and eicosapentaenoic acid groups were reduced by 6.77%, 27.75%, 23.18%, 19.46%, and 8.80%, respectively, compared to the stearic acid group, with the stearic acid group being significantly higher than the linoleic acid, -linolenic acid, and arachidonic acid groups. This indicates that adding 3% unsaturated long-chain fatty acids can reduce rumen protozoal protein content, possibly due to the negative effects of unsaturated long-chain fatty acids on rumen protozoa, which increase with degree of unsaturation. On one hand, unsaturated long-chain fatty acids inhibit protozoal growth, reducing protozoal density and numbers, and since protozoal biomass largely determines protozoal protein content, protein content decreases. On the other hand, protozoa cannot synthesize protein or utilize nitrogen sources to produce required amino acids; they rely primarily on proteins and peptides from microorganisms and feed as nitrogen sources. Unsaturated long-chain fatty acid addition inhibits protozoal bacterial engulfment capacity, hindering their nitrogen acquisition and thus reducing protozoal protein content.

Under *in vitro* culture conditions, the protozoal engulfment rate of bacteria in goat rumen generally decreased with increasing numbers of unsaturated bonds in long-chain fatty acids. The bacterial turnover rate was lowest in the linolenic acid group (0.68%) with the longest turnover time (146.92 h). In conclusion, -linolenic acid exhibits the best inhibitory effect on bacterial recycling due to protozoa engulfment in the rumen.

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