Profiles of Odd- and Branched-Chain Fatty Acids and Their Correlations With Rumen Fermentation Parameters, Microbial Protein Synthesis and Bacterial Populations Based on Pure Carbohydrate Incubation in Vitro

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Article: In vitro carbohydrate incubation and sample collection

The mixtures of fiber and starch were used as substrates for in vitro incubations and consisted of five different ratios [fiber : starch (F : S) = 0:100, 25:75, 50:50, 75:25 and 100:0]. Both fiber and starch were obtained from Macklin (Macklin Biochemical Co., Ltd, Shanghai, China). Around 1 g sample of each of the

Abstract

Background: The objectives of this study were to evaluate the profiles of odd- and branched-chain fatty acids (OBCFA; including C15:0, iso-C15:0, anteiso-C15:0, iso-C16:0, C17:0, iso-C17:0 and anteiso-C17:0) during pure carbohydrates incubation in vitro and whether they correlated with ruminal fermentation parameters, microbial crude protein (MCP) synthesis, and bacterial populations. The pure substrates containing five different ratios of fiber and starch (F:S; 0:100, 25:75, 50:50, 75:25 and 100:0) were incubated for 6 h, 12 h, 18 h and 24 h.

Results: Except iso-C17:0, OBCFA concentrations were interacted by F:S and incubation time. The highest concentration of total OBCFA was found in the fermented mixture after 24 h of incubation when the F:S = 0:100; while the lowest level was 1.65 mg/g DM produced after 6 h of incubation with F:S = 50:50. The concentrations of total volatile fatty acids (TVFA) and MCP remarkably decreased linearly as the inclusion of fiber in the substrates increased, as expected. The proportions of investigated cellulolytic bacteria in our study were increased linearly (or linearly and quadratically) while those of R. amylophilus and S. bovis were decreased as fiber inclusion increased. The correlation analysis indicated that iso-C16:0 concentration might have potential as a marker of productions of TVFA and MCP with p being 0.78 and 0.82 respectively. Compared to starch degrading bacteria, cellulolytic bacteria had more correlations with OBCFA profiles, and the strongest association was found on the population of R. flavefaciens with C15:0 concentration (p = 0.70).

Conclusions: Our study shows there might be scope for iso-C16:0 to predict rumen productions of VFA and MCP. Notedly, this is the first paper reporting linkage of OBCFA with rumen function based on pure carbohydrate in vitro incubation, which would avoid confounding interference from dietary protein and fat presence. However, more in-depth experiments are needed to substantiate the current findings.

Methods

In vitro incubation and sample collection

The mixtures of fiber and starch were used as substrates for in vitro incubations and consisted of five different ratios [fiber : starch (F : S) = 0:100, 25:75, 50:50, 75:25 and 100:0]. Both fiber and starch were obtained from Macklin (Macklin Biochemical Co., Ltd, Shanghai, China). Around 1 g sample of each of the
five mixed substrates was weighted and added into 150 mL culture flasks with rubber stoppers for 6, 12, 18 and 24 h of in vitro incubations. There were three replications for each treatment at each time point. Two runs were carried out within 2 weeks.

The ruminal fluid was obtained from three ruminally-fistulated Holstein cows fed a ration (Table 1) formulated according to the NRC (2001) [12] at 0800 am and 1600 pm daily. Rumen fluid was strained through four layers of cheesecloth and then transferred to a 4 L glass jar contained pre-warmed (39°C) buffer solution (rumen fluid : buffer = 1 : 2, v/v). The buffer solution including NH4HCO3, NaHCO3, Na2HPO4, KH2PO4, MgSO4·7H2O, CaCl2·2H2O, MnCl2·4H2O, CoCl2·6H2O, FeCl3·6H2O, C2H52HNO3Na, trypsin, C2H2N2O5SH-CH2H2O and Na2·S=2H2O was prepared according to Wang et al. [13]. Then 100 mL of mixed culture medium were pipetted into each flask, followed by the water-bath incubation at 39°C. The triplicate bottles only containing mixed culture medium were also incubated as the blank control. After 6, 12, 18 and 24 h of incubation, a total of 18 flasks (15 treatments and 3 blank) were taken out of the incubator and placed into ice to end the fermentation. Subsequently, approximate 5 mL of fermented inculom from each flask was subsampled for the measurements of pH value (Sartorius, pH-Meter PB-10, Goettingen, Germany), NH3-N and volatile fatty acids (VFA) concentrations. Then another 4 mL of inculom was snap frozen in liquid nitrogen and then stored at -80°C for further analysis of rumen bacterial populations. Finally, the remaining sample was freeze dried, weighed and then subjected to the analysis of OBCFA and MCP concentrations.

Measurement of odd- and branched-chain fatty acids

The OBCFA were determined according to the procedure described by Sukhija and Palmquist [14]. In brief, around 200 mg of freeze-dried fermentation mixture was weighed and added into each nylon-cap glass tube containing 2 mg of nonadecanoic acid (C19:0, internal standard; Macklin Biochemical Co., Ltd, Shanghai, China). Then the samples were treated with 3 ml of 5% methanolic HCl and incubated at 70°C for 2 h. After cooling to room temperature, 5 mL of 6% K2CO3 was added, followed by 2 mL of benzene and vortexed for 30 seconds. Following centrifugation at 1500 rpm for 5 min, the upper organic phase was transferred, dried with N2 and reconstituted in 1 mL of benzene for gas chromatograph (GC) analysis.

The methylated fatty acids were analyzed by Shimadzu GC analyzer (GC-2010, Shimadzu, Tokyo, Japan) with the flame ionization detector (FID) on an EquityTM-1 capillary column (15 m × 0.1 mm × 0.1 μm; Supelco, Inc., Sigma-Aldrich, 28039-U) with N2 as the carrier gas. The injector and detector were maintained at 280°C. Individual OBCFA was identified according to the peak and retention time of fatty acid standards including iso-C14:0, iso-C15:0, anteiso-C15:0, C15:0, iso-C16:0, iso-C17:0, anteiso-C17:0, C17:0 and iso-C18:0 (Larodan Fine Chemicals, Malmo, Sweden).

Analyses of ruminal fermentation parameters

The concentration of NH3-N was determined using the phenol-hypochlorite colorimetric method according to Broderick and Kang [15]. The VFA concentrations were measured by using gas chromatograph (GC-2010, Shimadzu Laboratory Supplies Co., Ltd, Shanghai, China) equipped with a flame-ionization detector and a capillary column (HP-Innowax, 19091N-133, Agilent Technologies, Santa Clara, CA), as described by Zhang et al. [3].

The production of MCP were estimated according to Hall and Herejk [16] in which trichloroacetic acid (TCA) was used to precipitate protein from microbial fermentations when pure carbohydrate substrates were fermented. Around 300 mg of freeze-dried fermentation residue was weighed, added into each 50 mL flask containing 10 mL of TCA (19.4%) which were then placed on the ice for 45 min. Following centrifugation at 7719 × g for 20 min, the whole flask content was filtered by Whatman 541 filter paper (Fisher Scientific, Atlanta, GA). Subsequently, the filtrate was filtered again through a Whatman GF/A glass fiber filter (Fisher Scientific, Atlanta, GA), using 2% TCA to rinse the flask. Both the Whatman 541 and GF/A filters were dried at 55°C overnight and then subjected for Kjeldahl analysis.

DNA extraction and qPCR analysis

Total DNA of ruminal microbes was extracted using the cetyl trimethyl ammonium bromide (CTAB) plus bead-beating method [17]. The air-dried DNA pellet was re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and was stored at -20°C until further processing [3]. The DNA purity (A260/280) was detected using a microplate spectrophotometer (SpectraMax 190, Sunnyvale, USA). Then the DNA samples were diluted to 10 ng/μL for Real-Time qPCR amplification. The qPCR primers used in this study (Table 2) were assembled according to previous studies [18,19,20,21] and obtained from Sangon Biotech Co. Ltd. (Shanghai, China). By following the manual instruction, the qPCR was performed using Takara SYBR® Premix Ex Taq. Synthesis Kit (Code No. RR420A, Takara, Dalian, China). The relative abundances of these bacteria were expressed as a proportion in total estimated bacterial 16S rDNA by the calculation of relative quantification = 2^[Ct target-Ct total bacteria], where Ct represents threshold cycle [22].

Statistical Analysis

The data were analyzed using PROC MIXED model in SAS (version 9.4, SAS Institute Inc., Cary, NC). The model included the fixed effects of different carbohydrate substrates (fiber : starch = 0.100, 25.75, 50:50, 75:25 and 100:0), incubation time (6, 12, 18 and 24 h), interaction effect (fiber/starch ratio × incubation time) and random effect of experimental run. Statistical significances were declared at P < 0.05. In order to investigate the relationships between OBCFA and fermentation parameters as well as selected bacterial populations, a Spearman rank correlation analysis was performed. A significant correlation was defined P < 0.05 and a strong correlation was defined when -0.5 > P > 0.5 and P < 0.05, where P is defined as the Spearman rank-order correlation coefficient. In this study, only significant correlations were shown.

Results

The ruminal OBCFA production in substrates with different ratios of fiber and starch in 24 h of incubation in vitro
Overall, seven OBCFA including iso-C15:0, anteiso-C15:0, C15:0, iso-C16:0, iso-C17:0 and anteiso-C17:0 were identified in our study (Table 3). Among them, anteiso-C15:0 and C15:0 were the top two abundant fatty acids, accounting for 17.6-23.6% and 15.4-38.1% of the total OBCFA, respectively. The total OBCFA concentration ranged from 2.3 to 7.1 mg/g DM in different residual samples after in vitro incubations.

All the OBCFA concentrations differed in different substrates across incubation times; however, the significant interactions between substrate type and incubation time (except iso-C17:0) might prohibit the comparison of main effects. The highest concentration of OBCFA was found in the incubated mixture after 24 h fermentation when the substrate was 100% pure starch; while the lowest level was 1.65 mg/g DM produced after 6 h of incubation with F:S = 50:50. The concentrations of C15:0, iso-C16:0, iso-C17:0, anteiso-C17:0 and C17:0 were linearly or/and quadratically changed ($P < 0.05$) as fiber proportion increased to 100% in the substrates. The OBCFA concentration was variated quadratically ($P < 0.05$), being decreased as fiber ratio increased to 50% and then increased gradually.

The concentrations of iso-C15:0, iso-C16:0, iso-C17:0, total iso-fatty acids (TIFA), total anteiso-fatty acids (TAFA), total branched-chain fatty acids (TBCFA) as well as OBCFA were linearly increased ($P < 0.05$), while the remaining OBCFA parameters were altered in both linear and quadratically fashions ($P < 0.05$) in all fermentation substrates across 24 h of ruminal incubation in vitro.

The ruminal fermentation parameters and MCP synthesis in substrates with different ratios of fiber and starch in 24 h of incubation in vitro

With the exception of acetate, total VFA (TVFA), pH and MCP synthesis, the rest parameters were significantly interacted by F:S ratio and incubation time ($P < 0.05$; Table 4). The molar proportions of propionate, isobutyrate, butyrate, isovalerate as well as acetate/propionate were changed in linear and quadratic fashions ($P < 0.05$) in different substrates; and the TVFA concentration remarkably decreased linearly ($P < 0.05$) as the inclusion of fiber in the substrates increased. The in vitro ruminal NH$_3$-N concentrations were significantly improved (linear or quadratic; $P < 0.05$) when then starch supply reduced in the substrates. The MCP synthesis was decreased linearly ($P < 0.05$) as F:S ratio increased. The VFA concentration was increased while pH value and NH$_3$-N concentrations decreased as incubation time increased ($P < 0.05$).

The bacterial population in substrates with different ratios of fiber and starch in 24 h of incubation in vitro

In the present study, three cellulyolytic bacteria (including $R$. albus, $R$. flavefaciens and $B$. fibrisolvens) and three amyloytic bacteria (including $S$. ruminantium, $R$. amylophilus and $S$. bovis) populations were measured using real-time qPCR system. Among them, $R$. flavefaciens (2.23-29.28%) and $R$. amylophilus (0.30-28.91%) were the top two abundant bacteria during 24 h of in vitro incubation. The third predominant bacterium - $S$. ruminantium, accounting for 0.42-1.11%, was not affected ($P > 0.05$) by F:S ratio and incubation time; whereas other bacterial populations had significant interactions of these two factors ($P < 0.05$). The proportions of investigated cellulyolytic bacteria in the present in vitro study were increased linearly (or linearly and quadratically; $P < 0.05$) while those of $R$. amylophilus and $S$. bovis were decreased ($P < 0.05$) as fiber inclusion increasing, as expected. For each kind of mixed carbohydrate substrates, the relative abundances of all the bacteria (except $S$. ruminantium) had marked reductions across 24 h of incubation on most occasions, either in linear or linear and quadratic fashions ($P < 0.05$).

Correlation analysis between OBCFA production and fermentation parameters and bacterial populations during 24 h of incubation in vitro

To test the interaction between ruminal fermentation products and OBCFA synthesis, a further analysis was investigated based on Spearman's rank correlation using the corresponding variables (Table 6) during 24 h of incubation of pure carbohydrates in vitro.

In general, more correlations were observed between the molar proportion of acetate and OBCFA production than between other individual VFA molar proportion and OBCFA production (Figure 1). The acetate molar proportion negatively correlated ($P < 0.05$; $\rho = -0.32 \sim -0.69$) with all OBCFA production. Within these correlations, ten of them were relatively stronger ($\rho < -0.50$). The molar proportion of propionate positively correlated with concentrations of C15:0 ($P < 0.05$; $\rho = 0.77$), TC15 (TC15 = iso-C15:0 + anteiso-C15:0 + C15:0; $P < 0.05$; $\rho = 0.67$) and TOCFA (TOCF = C15:0 + C17:0; $P < 0.05$; $\rho = 0.73$), while those of isobutyrate ($P < 0.05$; $\rho = -0.60$) and butyrate ($P < 0.05$; $\rho = 0.65$) correlated with isoC16:0 concentration. The concentration of TVFA had positive relationships with all OBCFA parameters ($P < 0.05$; $\rho = 0.49 \sim 0.78$), with the exceptions of C15:0, TC15 and TOCFA ($P > 0.05$).

Except C15:0, the concentrations of OBCFA were observed to be negatively related to NH$_3$-N ($P < 0.05$; $\rho = -0.39 \sim -0.73$) and positively correlated to MCP synthesis ($\rho < 0.05$; $\rho = 0.37 \sim 0.82$). By using the measured MCP synthesis data, we found it had relatively stronger relationships with iso-C16:0 ($\rho = 0.82$) and anteiso-C17:0 ($\rho = 0.66$) concentrations, suggesting these two fatty acids, especially for iso-C16:0, had potential capability to predict microbial flow in the rumen.

We further investigated the potential correlations between OBCFA and bacterial populations (Figure 2). More correlations were observed between ruminal OBCFA production and cellulyolytic bacteria than between OBCFA and starch-degrading bacteria. Of note, the relative abundances of $R$. albus ($P < 0.05$; $\rho = 0.58$) and $R$. flavefaciens ($P < 0.05$; $\rho = 0.70$) positively correlated with C15:0, while that of $B$. fibrisolvens negatively correlated with anteiso-C15:0 ($P < 0.05$; $\rho = -0.57$) and iso-C16:0 ($P < 0.05$; $\rho = -0.56$). Although significanc detected, the correlations with $\rho$ between OBCFA and $S$. ruminantium, $R$. amylophilus and $S$. bovis populations were all lower than 0.50.

Discussion

The proportions of anteiso-C15:0 and C15:0 observed in the current study are largely consistent with those in the fermented mixtures (aneiso-C15:0, 24.7-29.6%; C15:0, 24.9-27.9%) after 21 h of incubation in vitro with four types of dairy diet as substrates [1] as well as in the rumen bacteria (aneiso-C15:0, 23.7%; C15:0, 25.7%) collected from Holstein cows under different feeding regimes [23, 24]. Vlaeminck et al. [25] reported a close relationship ($r = -0.77$) between anteiso-C15:0 concentration and dietary forage inclusion. However, the anteiso-C15:0 concentration observed in this study was minimally changed as
fiber addition increased during *in vitro* incubation. Generally, the odd-chain fatty acids are formed using propionate as primer [4]. As for the branched-chain fatty acids, the iso-fatty acids are produced from isobutyrate and isovalerate while antiso-fatty acids are originated from 2-methylbutyrate in ruminants, as summarized by Vlaeminck et al. [11]. The generation of these short chain fatty acids is not only influenced by dietary strategy [3, 26], but also highly depending on ruminal environment and physiological conditions of cows [27]. Indeed, studies regarding ruminal OBCFA productions in cows fed rations with different ratios of forage and concentrate have shown contrasting results. With increasing supplementary levels of forage in the diets, total OBCFA production has been reported to be linearly increased in the study of Vlaeminck et al. [25], whereas quadratically changed in rumen contents at 6 h after the morning feeding as observed by Zhang et al. [3] which is partially in line with our data. As known, the OBCFA, especially BCFA, mainly derive from bacteria and meanwhile are the unique components of cell membrane lipids in kinds of bacteria [4]; and exist in rumen protozoa as well [23, 24]. Therefore, the increasing OBCFA production obtained in the present study might indicate the microorganisms were keeping a strong reproducibility and proliferation during the whole *in vitro* incubation, which would be beneficial for microbial metabolic process and fermentation activities. However, the changes in ruminal OBCFA production within 12–17 h after feeding found in our preliminary study [28] and an earlier report of Vlaeminck et al. [2] mainly disagree with our results. These might be due to the characteristics of ruminal *in vitro* culture system in which the fermentation products (eg. VFAs) could not outflow nor be absorbed by rumen mucosal epithelia, which might contribute more to OBCFA syntheses and accumulation in the mixtures within 24 h of fermentation in our study.

The observed VFA profiles in the present study are in accordance with recent studies of Wang et al. [22] and Li et al. [29] who all confirmed that increased dietary forage proportion could decrease TVFA in the rumen of dairy cows. Increasing proportion of fermented carbohydrate, such as starch, would enhance the microorganisms to capture released NH$_3$-N to synthesize ruminal microbial protein, as illustrated by Li et al. [29], which might partly explain our results. As well known, the short chain fatty acids (eg. VFA) produced during rumen fermentation are readily absorbed by rumen epithelial papilla in ruminants [30].

However, the failure of VFA absorption in batch *in vitro* system would result in higher accumulation of VFA and decreased pH values as incubation time increased, which is also supported by our findings. Furthermore, the reduced NH$_3$-N concentrations might suggest these released nutrients were utilized by microorganisms, which paralleled with the increased production of MCP within 24 h of incubation *in vitro* in our study.

Increasing dietary forage proportion promotes the growth of cellulose-degrading bacteria and meanwhile reduced the relative importance of amylolytic bacteria in the rumen [22, 31], being consistent with our observations. However, the observed reductions of the relative abundances of all the bacteria (except *S. ruminantium*) across 24 h of incubation indicated microbial lysis and decomposition occurred over incubation [32] in a batch *in vitro* system. This phenomenon is in part supported by the considerably decreased microbial activities in rumen fluid stored at anaerobically at 39°C for 24 h comparing with fresh inoculum [33].

Rumen fermentation products, namely short chain fatty acids are usually regarded as precursors in OBCFA synthesis [25]. The quantities of OBCFA have been reported to potentially predict microbial growth [2] since these fatty acids, especially for branched-chain fatty acids, are the major components in bacterial membrane lipids [4]. However, studies exploring rumen VFA and OBCFA relationships have shown mixed results. Even though, our results in part agreed with previous findings achieved from an *in vitro* incubation with nine mixed dairy cow rations [1] in which the molar proportion of acetate was found to have negative associations with C15:0 and iso-C17:0; and that of propionate had positive relationships with C15:0. However, French et al. [6] found ruminal OBCFA response was minor following infusion of large amounts of acetate and propionate. Then they concluded that extracellular VFA concentrations might not be responsible for the variations of rumen OBCFA. Furthermore, the rumen OBCFA profiles have been reported to be largely determined by relative abundance of specific microbial strains populations rather than OBCFA synthesis related to the availability of primers [11, 34].

The correlations of OBCFA concentrations with NH$_3$-N and MCP should be largely interpreted with the utilization and incorporation of ammonia to produce MCP by rumen microorganisms that enriching OBCFA in their cell membranes [4]. Similarly, Zhang et al. [3] showed there were negative relationships between NH$_3$-N and OBCFA in the rumen contents in Holstein cows. Even though the measured MCP synthesis was not available in previous literatures [2, 7], these authors demonstrated that sum content of OBCFA or some specific OBCFA (eg. C13:0, C15:0, iso-C15:0 and C17:0) was closely related to microbial markers, such as uracil, purine bases [2] and pyrimidine [7].

Microbial shift driven by altered dietary or environmental conditions [35, 36] might lead to OBCFA variations in the rumen. Based on the measured data in the pure culture studies, the OBCFA compositions are always species-specific [9, 37, 38], or even varies widely in strains [10, 38]. Some strains of *R. albus* spp. (eg. *R. albus* 7) contain high amount of linear odd-chain fatty acids [39] and this is also supported by our finding on the positive relationships between concentrations of C15:0 and TOCFA and *R. albus* relative abundance. Earlier several studies have reported that iso-C15:0 is the most predominant OBCFA in the pure cultured strains of *R. flavefaciens* (*R. flavefaciens* FD1 and CF4), accounting for 37–41% of total fatty acids [34, 39]. Combining these results along with the positive correlation between iso-C15:0 and *R. flavefaciens* in our experiment indicated that more abundance of this bacteria might produce high level of isoacids in the rumen. Increasing dietary forage proportion would lead to improved growth of cellulolytic bacteria such as *R. flavefaciens* [22, 40], and expectedly, result in higher production iso-C15:0 in the rumen [3]. However, the strongest association we found in this study was on the population of *R. flavefaciens* with C15:0 concentration, which seemed to be discrepant with measured cellular fatty acid compositions in pure culture studies [34, 39]. As summarized by Vlaeminck et al. [11], *B. fibrisolvens* had heterogeneous profile of OBCFA, which might partly explain their negative relations in our study. The observed weak to moderate associations between amylolytic bacteria and OBCFA in the present study are similar with the results reported by Zhang et al. [3] who found negative correlation between *S. ruminantium* with anteiso-C17:0 (p = 0.21, P < 0.05) whereas positive relationship between *S. bovis* and C15:0 (p = 0.44, P < 0.05) and total production of OBCFA (p = 0.37, P < 0.05) in 108 rumen samples obtained from dairy cows fed rations with different ratios of forage and concentrate.

**Conclusions**

Incubating pure carbohydrate with different ratios of fiber and starch produced different amounts of OBCFA during 24 h of incubation *in vitro*. Except iso-C17:0, other individual OBCFA productions were all interacted by F:S ratio and incubation time. The highest concentration of total OBCFA was found in the
incubated mixture after 24 h of fermentation when the F:S = 0:100; while the lowest level was 1.65 mg/g DM produced after 6 h of incubation with F:S = 50:50. The correlation analysis indicated that the OBCFA concentrations were closely related to rumen VFA, MCP and bacterial populations. In particular, the iso-C16:0 concentration might have potential as a marker of productions of total VFA and MCP with ρ being 0.78 and 0.82 respectively. Compared to starch degrading bacteria, cellulolytic bacteria had relatively strong correlations with OBCFA profiles. However, to achieve a final accurate conclusion on the linkage of OBCFA with rumen fermentation profiles, more in-depth experiments are needed to substantiate the current findings.

**Abbreviations**

anteiso-C15 0:12-methyltetradecanoic acid; anteiso-C17:0:14-methylhexadecanoic acid; C15:0: pentadecanoic acid; C17:0: heptadecanoic acid; DM: dry matter; F:S: fiber:starch; iso-C15:0:13-methyltetradecanoic acid; iso-C16:0:14-methylpentadecanoic acid; iso-C17:0:15-methylhexadecanoic acid; MCP: microbial crude protein; NH\textsubscript{3}-N: ammonia nitrogen; OBCFA: odd- and branched-chain fatty acids; TCA: trichloroacetic acid; TIFA: total iso-fatty acids; TAFA: total anteiso-fatty acids; TOCFA: total odd-chain fatty acids; TBCFA: total branched-chain fatty acids; TVFA: total volatile fatty acids; VFA: volatile fatty acids.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors provide their consent to this publication.

**Availability of data and material**

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare no competing financial interest.

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**Authors’ contributions**

HX and HW conceived, designed the whole experiment and wrote the manuscript; XL, XJ, CL, QF, SZ, YL, YS, HW and YZ conducted the experiment, did laboratory analyses and data analysis.

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Tables

Table 1

| Ingredients (%) | Chemical composition |
|-----------------|----------------------|
| Chinese wildrye | 43.0 NE\textsuperscript{2} \text{, MJ/kg DM} 5.4 |
| Corn silage     | 16.0 Crude protein, %DM 14.4 |
| Corn            | 13.0 Neural detergent fiber, %DM 49.2 |
| Wheat bran      | 4.0 Acid detergent fiber, %DM 30.6 |
| Molasses        | 1.0 Ca, %DM 0.6 |
| Soybean meal    | 3.0 P, %DM 0.4 |
| Dried distillers grain | 5.0 |
| Cottonseed meal | 2.0 |
| Corn gluten feed| 7.5 |
| Corn germ meal  | 5.0 |
| Premix \textsuperscript{1} | 0.5 |
| Total           | 100.0 |

\textsuperscript{1} Contained the following per kg of the premix: V\textsubscript{A} 8000000 IU, V\textsubscript{D} 700000 IU, V\textsubscript{E} 10000 IU, Fe 1600 mg, Cu 1500 mg, Zn 10000 mg, Mn 3500 mg, Se 80 mg, I 120 mg, Co 50 mg.

\textsuperscript{2} NE\textsubscript{L} is calculated value and other nutrient levels are measured values.

Table 2

| Primer sequence (5’-3’) | Product size (bp) | Reference |
|-------------------------|-------------------|-----------|
| General bacteria        | F:CGGCAACGAGCGCAACCC R:CCATTGTGACGCTGTCGTC | 130 | Denman and McSweeney (2006) |
| Ruminococcus albus      | F:GTTTTAGGATTGTAAACCTCTTGTTT R:CCTAATATCTGCGGTACGC | 273 | Yang (2007) |
| Ruminococcus flavifaciens | F:GATGCCGCGTGGAAGGAAGAAG R:CATTTCACCGCTACACCAGGAA | 278 | Yang (2007) |
| Butyribrio fibrisolvens | F:TAACATGAGTTTTATCGCTGGCTC R:CGTTTACCTGCCGCTGGC | 113 | Yang (2007) |
| Selenomonas ruminantium | F:CAATAAGCATTTCCCGCTGGG R:TTCCTTACATGTACGCTGGG | 138 | Stevenson and Weimer (2007) |
| Streptococcus bovis      | F:TTCTAGAGATAGAACGGATTTTCTTCCGG R:ATGATGAACTAATACATGAGGTT | 127 | Stevenson and Weimer (2007) |
| Ruminobacter amylophilus | F:CTGGGAGCTGGGCTGAAT R:CATCTGAATGCGACTGGTTT | 100 | Khafipour et al. (2009) |
Table 3

Effect of different ratios of fiber and starch on ruminal OBCFA production (mg/g DM) in 24 h of incubation in vitro

| Time h | iso-C15:0 | anteiso-C15:0 | C15:0 | iso-C16:0 | anteiso-C16:0 | C17:0 | TC15 | TC17 | TIFA | TFA | TOC |
|--------|-----------|---------------|-------|-----------|---------------|-------|------|------|------|-----|-----|
| F:S = 0:100<sup>a</sup> | 6 | 0.22 | 0.54 | 0.56 | 0.31 | 0.31 | 0.37 | 0.35 | 1.31 | 1.02 | 0.84 | 0.90 | 0.90 |
| | | 12 | 0.23 | 0.58 | 0.51 | 0.33 | 0.36 | 0.38 | 0.27 | 1.29 | 1.00 | 0.91 | 0.96 | 0.78 |
| | | 18 | 0.24 | 0.60 | 0.55 | 0.38 | 0.39 | 0.46 | 0.31 | 1.39 | 1.17 | 1.01 | 1.06 | 0.87 |
| | | 24 | 0.31 | 0.76 | 0.69 | 0.48 | 0.47 | 0.80 | 0.43 | 1.75 | 1.71 | 1.26 | 1.56 | 1.12 |
| F:S = 25:75 | 6 | 0.19 | 0.43 | 0.33 | 0.25 | 0.32 | 0.30 | 0.32 | 0.95 | 0.94 | 0.76 | 0.73 | 0.65 |
| | | 12 | 0.21 | 0.56 | 0.42 | 0.26 | 0.33 | 0.35 | 0.25 | 1.19 | 0.92 | 0.80 | 0.90 | 0.66 |
| | | 18 | 0.24 | 0.64 | 0.62 | 0.34 | 0.41 | 0.49 | 0.33 | 1.50 | 1.24 | 0.99 | 1.13 | 0.95 |
| | | 24 | 0.30 | 0.75 | 0.59 | 0.42 | 0.45 | 0.64 | 0.40 | 1.64 | 1.48 | 1.17 | 1.39 | 0.99 |
| F:S = 50:50 | 6 | 0.14 | 0.29 | 0.34 | 0.17 | 0.26 | 0.24 | 0.22 | 0.76 | 0.72 | 0.57 | 0.53 | 0.56 |
| | | 12 | 0.21 | 0.57 | 0.54 | 0.26 | 0.35 | 0.44 | 0.24 | 1.32 | 1.03 | 0.82 | 1.01 | 0.75 |
| | | 18 | 0.25 | 0.59 | 0.61 | 0.23 | 0.35 | 0.40 | 0.23 | 1.45 | 0.98 | 0.83 | 0.98 | 0.85 |
| | | 24 | 0.33 | 0.76 | 0.61 | 0.38 | 0.40 | 0.83 | 0.29 | 1.70 | 1.52 | 1.11 | 1.58 | 0.91 |
| F:S = 75:25 | 6 | 0.18 | 0.40 | 0.32 | 0.18 | 0.32 | 0.25 | 0.23 | 0.91 | 0.81 | 0.68 | 0.66 | 0.55 |
| | | 12 | 0.28 | 0.71 | 1.03 | 0.30 | 0.35 | 0.34 | 0.27 | 2.01 | 0.95 | 0.92 | 1.04 | 1.30 |
| | | 18 | 0.37 | 0.76 | 1.10 | 0.25 | 0.41 | 0.31 | 0.31 | 2.24 | 1.04 | 1.03 | 1.08 | 1.42 |
| | | 24 | 0.37 | 0.82 | 1.04 | 0.33 | 0.44 | 0.35 | 0.44 | 2.24 | 1.24 | 1.15 | 1.18 | 1.46 |
| F:S = 100:0 | 6 | 0.14 | 0.31 | 0.37 | 0.11 | 0.32 | 0.26 | 0.24 | 0.82 | 0.81 | 0.57 | 0.57 | 0.61 |
| | | 12 | 0.23 | 0.63 | 0.94 | 0.18 | 0.33 | 0.28 | 0.29 | 1.80 | 0.90 | 0.74 | 0.91 | 1.23 |
| | | 18 | 0.29 | 0.65 | 1.15 | 0.15 | 0.35 | 0.25 | 0.30 | 2.09 | 0.91 | 0.79 | 0.91 | 1.45 |
| | | 24 | 0.29 | 0.64 | 1.25 | 0.16 | 0.38 | 0.26 | 0.32 | 2.18 | 0.95 | 0.83 | 0.90 | 1.56 |

SEM

| Time | iso-C15:0 | anteiso-C15:0 | C15:0 | iso-C16:0 | anteiso-C16:0 | C17:0 | TC15 | TC17 | TIFA | TFA | TOC |
|------|-----------|---------------|-------|-----------|---------------|-------|------|------|------|-----|-----|
| F:S  | <.0001    | <.0001        | <.0001| <.0001    | <.0001        | <.0001| <.0001| <.0001| <.0001| <.0001| <.0001|
| Time | <.0001    | <.0001        | <.0001| <.0001    | <.0001        | <.0001| <.0001| <.0001| <.0001| <.0001| <.0001|
| F:S x Time | 0.0003 | 0.0001 | <.0001 | <.0001 | <.0001 | 0.13 | 0.0006 | <.0001 | <.0001 | <.0001 | <.0001 |

Polynomial contrast

| Time | iso-C15:0 | anteiso-C15:0 | C15:0 | iso-C16:0 | anteiso-C16:0 | C17:0 | TC15 | TC17 | TIFA | TFA | TOC |
|------|-----------|---------------|-------|-----------|---------------|-------|------|------|------|-----|-----|
| F:S  | <.0001    | <.0001        | <.0001| <.0001    | <.0001        | <.0001| <.0001| <.0001| <.0001| <.0001| <.0001|
| Time | <.0001    | <.0001        | <.0001| <.0001    | <.0001        | <.0001| <.0001| <.0001| <.0001| <.0001| <.0001|
| F:S x Time | 0.0003 | 0.0001 | <.0001 | <.0001 | <.0001 | 0.13 | 0.0006 | <.0001 | <.0001 | <.0001 | <.0001 |

<sup>a</sup>TBCFA = iso-C15:0 + iso-C16:0 + iso-C17:0 + anteiso-C15:0 + anteiso-C17:0.<sup>b</sup>TAFA = anteiso-C15:0 + anteiso-C17:0.<sup>c</sup>OBCFA = iso-C15:0 + anteiso-C15:0 + C15:0 + iso-C16:0 + iso-C17:0.<sup>d</sup>TIFA = iso-C15:0 + iso-C16:0 + iso-C17:0.<sup>e</sup>TIFA = iso-C15:0 + iso-C16:0 + iso-C17:0.<sup>f</sup>F:S = Fiber : Starch.
## Table 4

Effect of different ratios of fiber and starch on ruminal fermentation parameters and MCP\(^1\) production in 24 h of incubation in vitro

| Time, h | acetate | propionate | isobutyrate | butyrate | isovalerate | TVFA | acetate/propionate | NH\(_3\)-N | pH | MCP |
|--------|---------|------------|-------------|----------|-------------|------|-------------------|---------|----|-----|
|        | mmol/mol| mmol/mol   | mmol/mol    | mmol/mol | mmol/mol    | mmol/L|                   | mg/dL   |    |     |
| F:S = 0:100\(^2\) |         |            |             |          |             |       |                   |         |    |     |
| 6      | 553.91  | 220.07     | 8.78        | 206.38   | 10.86       | 55.48 | 2.52              | 1.38    | 6.50| 119.91 |
| 12     | 509.98  | 251.98     | 7.95        | 211.52   | 10.85       | 73.17 | 2.02              | 0.45    | 6.41| 119.99 |
| 18     | 510.62  | 231.21     | 8.82        | 229.77   | 12.41       | 88.11 | 2.22              | 0.31    | 6.33| 133.81 |
| 24     | 496.11  | 241.07     | 9.21        | 232.46   | 13.28       | 85.78 | 2.06              | 2.56    | 6.26| 147.41 |
| F:S = 25:75 |         |            |             |          |             |       |                   |         |    |     |
| 6      | 546.64  | 222.72     | 9.24        | 209.36   | 12.04       | 53.06 | 2.45              | 4.94    | 6.64| 93.18  |
| 12     | 510.91  | 254.64     | 8.70        | 214.44   | 11.31       | 59.30 | 2.01              | 4.00    | 6.58| 94.77  |
| 18     | 507.36  | 258.18     | 8.44        | 206.93   | 11.53       | 78.00 | 1.97              | 0.69    | 6.48| 99.35  |
| 24     | 495.45  | 251.38     | 7.95        | 226.18   | 11.43       | 78.76 | 1.97              | 2.73    | 6.39| 119.55 |
| F:S = 50:50 |         |            |             |          |             |       |                   |         |    |     |
| 6      | 547.20  | 221.40     | 9.30        | 209.56   | 12.54       | 52.89 | 2.48              | 7.83    | 6.71| 97.20  |
| 12     | 521.25  | 242.46     | 8.68        | 215.41   | 12.20       | 59.24 | 2.15              | 3.12    | 6.62| 104.81 |
| 18     | 521.24  | 241.88     | 7.88        | 209.79   | 10.70       | 68.34 | 2.16              | 4.20    | 6.55| 108.03 |
| 24     | 506.79  | 240.04     | 8.58        | 224.62   | 11.90       | 72.77 | 2.11              | 4.03    | 6.49| 111.88 |
| F:S = 75:25 |         |            |             |          |             |       |                   |         |    |     |
| 6      | 532.98  | 220.81     | 10.61       | 221.13   | 14.47       | 44.06 | 2.41              | 11.19   | 6.78| 76.22  |
| 12     | 510.73  | 244.97     | 9.51        | 222.28   | 12.51       | 52.97 | 2.09              | 6.33    | 6.70| 99.93  |
| 18     | 504.93  | 260.63     | 8.48        | 215.54   | 10.42       | 68.48 | 1.94              | 3.35    | 6.55| 107.91 |
| 24     | 485.84  | 271.82     | 8.96        | 221.98   | 11.40       | 69.14 | 1.79              | 2.07    | 6.48| 108.38 |
| F:S = 100:0 |       |            |             |          |             |       |                   |         |    |     |
| 6      | 536.13  | 240.41     | 13.31       | 192.16   | 17.99       | 35.10 | 2.23              | 17.05   | 6.85| 69.51  |
| 12     | 522.14  | 273.45     | 11.44       | 177.51   | 15.45       | 45.20 | 1.91              | 10.62   | 6.80| 76.89  |
| 18     | 498.46  | 318.17     | 9.78        | 160.53   | 13.07       | 54.86 | 1.57              | 7.06    | 6.67| 86.18  |
| 24     | 493.44  | 323.82     | 11.62       | 157.32   | 13.79       | 56.76 | 1.53              | 7.83    | 6.61| 85.65  |
| SEM    | 6.699   | 5.429      | 0.429       | 6.048    | 0.446       | 3.243 | 0.061             | 0.737   | 0.040| 5.065  |
| \(P\)  | F:S     | < .0001    | < .0001     | < .0001  | < .0001     | < .0001| < .0001           | < .0001 | < .0001| < .0001 |
|        | Time    | < .0001    | < .0001     | < .0001  | < .0001     | < .0001| < .0001           | < .0001 | < .0001| < .0001 |
|        | F:S x Time | 0.68    | < .0001     | 0.02     | 0.001       | < .0001| 0.56              | 0.007   | < .0001| 0.99  |
| Polynomial contrast - F:S | Linear | < .0001    | < .0001     | < .0001  | < .0001     | < .0001| < .0001           | < .0001 | < .0001| < .0001 |
|         | Quadratic | 0.36    | < .0001     | < .0001  | < .0001     | < .0001| 0.05              | < .0001 | 0.001| 0.12  |
| Polynomial contrast - Time | Linear | < .0001    | < .0001     | 0.0002   | 0.38        | < .0001| < .0001           | < .0001 | < .0001| < .0001 |
|         | Quadratic | 0.01    | < .0001     | 0.0002   | 0.17        | < .0001| 0.004             | < .0001 | 0.88 | 0.90  |

\(^1\)MCP = microbial crude protein synthesis; \(^2\)F : S = Fiber : Starch.
Table 5

| Time (h) | F:S = 0:100 | F:S = 25:75 | F:S = 50:50 | F:S = 75:25 | F:S = 100:0 |
|---------|-------------|-------------|-------------|-------------|-------------|
| 6       | 0.19        | 0.40        | 0.16        | 0.30        | 0.17        |
| 12      | 0.10        | 0.03        | 0.23        | 0.27        | 0.22        |
| 18      | 0.06        | 0.07        | 0.13        | 0.34        | 0.70        |
| 24      | 0.09        | 0.07        | 0.09        | 0.56        | 1.48        |

Bacteria population was measured as a proportion of the total estimated rumen bacterial 16S rRNA gene (relative quantification = $2^{-\Delta CT_{bacteria}-\Delta CT_{total bacteria}}$); F:S = Fiber : Starch.
| Table 6 |
|------------------------------|
| Summary statistics of variables utilized for the correlation analysis |

| Variable                          | Mean  | Standard deviation | Minimum | Maximum |
|-----------------------------------|-------|--------------------|---------|---------|
| OBCFA profile, mg/g DM            |       |                    |         |         |
| iso-C15:0                         | 0.40  | 0.12               | 0.16    | 0.70    |
| anteiso-C15:0                     | 0.94  | 0.25               | 0.39    | 1.44    |
| C15:0                             | 1.09  | 0.56               | 0.46    | 2.48    |
| iso-C16:0                         | 0.42  | 0.12               | 0.18    | 0.64    |
| iso-C17:0                         | 0.58  | 0.10               | 0.33    | 0.79    |
| anteiso-C17:0                     | 0.62  | 0.22               | 0.34    | 1.46    |
| C17:0                             | 0.48  | 0.11               | 0.29    | 0.84    |
| TC15<sup>1</sup>                  | 2.43  | 0.88               | 1.05    | 4.25    |
| TC17<sup>2</sup>                  | 1.67  | 0.32               | 0.99    | 2.66    |
| TIFA<sup>3</sup>                  | 1.39  | 0.27               | 0.75    | 2.07    |
| TAFA<sup>4</sup>                  | 1.56  | 0.39               | 0.73    | 2.77    |
| TOCFA<sup>5</sup>                 | 1.57  | 0.62               | 0.79    | 3.09    |
| TBCFA<sup>6</sup>                 | 2.95  | 0.63               | 1.48    | 4.64    |
| OBCFA<sup>7</sup>                 | 4.52  | 1.10               | 2.30    | 7.10    |

Ruminal fermentation parameters and MCP synthesis

| Variable                          | Mean  | Standard deviation | Minimum | Maximum |
|-----------------------------------|-------|--------------------|---------|---------|
| acetate, mmol/mol                 | 515.61| 21.24              | 482.51  | 572.28  |
| propionate, mmol/mol              | 251.55| 29.00              | 212.15  | 340.09  |
| isobutyrate, mmol/mol             | 9.36  | 1.50               | 6.83    | 13.77   |
| butyrate, mmol/mol                | 208.24| 22.43              | 148.56  | 244.17  |
| isovalerate, mmol/mol             | 12.51 | 1.91               | 9.44    | 18.41   |
| TVFA, mmol/L                      | 62.57 | 14.75              | 33.67   | 89.64   |
| acetate/propionate                | 2.08  | 0.28               | 1.43    | 2.59    |
| NH<sub>3</sub>-N, mg/dL           | 5.09  | 4.29               | 0.30    | 18.54   |
| pH                                | 6.57  | 0.16               | 6.19    | 6.90    |
| MCP, g/kg DM                      | 160.73| 22.04              | 110.07  | 205.65  |

Ruminal bacterial population

| Species                           | Mean  | Standard deviation | Minimum | Maximum |
|-----------------------------------|-------|--------------------|---------|---------|
| *R. albus*, <span class="footnote">10<sup>-2</sup></span>% | 0.29  | 0.36               | 0.02    | 2.17    |
| *R. flavefaciens*, 10<sup>-2</sup>%       | 3.60  | 5.63               | 0.10    | 28.40   |
| *B. fibrisolvens*, %               | 10.89 | 7.37               | 1.78    | 31.86   |
| *S. ruminantium*, %                | 0.68  | 0.32               | 0.25    | 1.51    |
| *R. amylophilus*, %                | 11.84 | 9.57               | 0.27    | 44.44   |
| *S. bovis*, 10<sup>-2</sup>%        | 0.23  | 0.21               | 0.04    | 1.10    |

<sup>1</sup> TC15 = iso-C15:0 + anteiso-C15:0 + C15:0; <sup>2</sup> TC17 = iso-C17:0 + anteiso-C17:0 + C17:0; <sup>3</sup> TIFA = iso-C15:0 + iso-C16:0 + iso-C17:0; <sup>4</sup> TAFA = anteiso-C15:0 + anteiso-C17:0; <sup>5</sup> TOCFA = C15:0 + C17:0; <sup>6</sup> TBCFA = iso-C15:0 + iso-C16:0 + iso-C17:0 + anteiso-C15:0 + anteiso-C17:0; <sup>7</sup> OBCFA = iso-C15:0 + anteiso-C15:0 + C15:0 + iso-C16:0 + iso-C17:0 + anteiso-C17:0 + C17:0.

**Figures**
Correlation analysis on ruminal fermentation parameters, MCP and OBCFA concentrations after 6, 12, 18 and 24 h of incubation in vitro. *Color in round shape represents significant correlations (P < 0.05) and black square means -0.50 > ρ > 0.50.
Correlation analysis on ruminal bacterial populations and OBCFA concentrations during 24 h of incubation in vitro. *Color in round shape represents significant correlations (P < 0.05) and black square means -0.50 > ρ > 0.50.

Figure 2