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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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 ρ 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 (ρ = 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.

Background

Odd- and branched-chain fatty acids [**OBCFA**; including pentadecanoic acid (C15:0), 13-methyltetradecanoic acid (iso-C15:0), 12-methyltetradecanoic acid (anteiso-C15:0), 14-methylpentadecanoic acid (iso-C16:0), heptadecanoic acid (C17:0), 15-methylhexadecanoic acid (iso-C17:0) and 14-methylhexadecanoic acid (anteiso-C17:0)] are promising for predicting ruminal fermentation and microbial matter leaving the rumen [1, 2, 3], because they are dominant components in microbial membrane lipids [4]. Theoretically, these fatty acids are produced from 2-carbon elongation of propionate (C15:0 or C17:0), isobutyrate (iso-C14:0 or iso-C16:0), isovalerate (iso-C15:0 or iso-C17:0) and 2-methyl-butyrate (anteiso-C15:0 or anteiso-C17:0) with the incorporation of malonyl-CoA fatty acid synthetase [4, 5]. Therefore, it is not surprising to have several studies reporting close relationships between OBCFA and rumen VFA patterns both *in vitro* [1] and *in vivo* [3]. For instance, the bacterial OBCFA has been testified to explain 80% of the variations in acetate, propionate and butyrate under an *in vitro* culture system [1]. However, French et al. [6] demonstrated that infusion of large amounts of VFA failed to alter OBCFA proportions in the rumen in mid-lactation dairy cows. These mentioned studies have used a variety of natural feedstuff containing amount of ether extract [1, 6]. And dietary fat provision was always one of the major components in OBCFA concentrations in bacteria [1]. So, it is likely that the presence of dietary fat might be able to change OBCFA concentrations by changing microbial makeup in the rumen. As a result, these findings could not be attributed clearly to individual constitute and may not extend well to different feedstuffs.

Since most OBCFA arise from microbial membrane lipids [4], increased concentrations of these fatty acids could indicate increased microbial yield. Vlaeminck et al. [2], investigating OBCFA as rumen microbial marker, obtained that ratios of purine bases : OBCFA and uracil : OBCFA were constant, being 1.16 and 0.34 in mixed rumen bacteria of dairy cows, respectively. Similarly, our preliminary study confirmed that ruminal OBCFA concentrations, especially for odd-chain fatty acids as well as isomers with 15 carbon, significantly correlated with pyrimidine (R^2 = 0.61), cytosine (R^2 = 0.57) and total nucleic acid bases (R^2 = 0.54) in dairy cows [7]. Although these nucleic acids are microbial origin and widely used to quantify bacterial protein yield, they are not reliable completely due to their ratio with nitrogen content considerably varied between solid-associated bacteria and liquid-associated bacteria in the rumen [8]. To the best of our knowledge, none of studies have measured the true synthesis of microbial crude protein (MCP) in these experiments; therefore, the relationship between OBCFA concentration and MCP yield has not been well determined.

In addition, different bacteria bear different OBCFA compositions in the rumen [9, 10]. As well reviewed by Vlaeminck et al. [11], fibrolytic bacteria (eg. *Ruminococcus flavefaciens* and *Ruminococcus albus*) usually enriched in iso-fatty acids while starch degrading microorganisms (eg. *Selenomonas ruminantium*, *Ruminobacter amylophilus* and *Streptococcus bovis*) contained relatively high amount of linear odd-chain fatty acids and low levels of branched-chain fatty acids. In comparison, strains of *Butyrivibrio* have more heterogenous profile of OBCFA [10]. Regarding the linkage of OBCFA concentrations with bacterial abundance in the rumen content, little information is available, although data from our preliminary work indicated fatty acid of C17:0 might have potentials to quantify cellulolytic bacterial population in mixed rumen bacteria. However, to build up a solid conclusion, more in-depth studies are warrant.

In our study, pure carbohydrates (fiber and starch) were selected as substrates to avoid confounding effects of dietary fat on OBCFA synthesis during rumen fermentation. We hypothesized the profiles of OBCFA would be different in substrates with different ratios of fiber and starch and they might be correlated with rumen function during rumen fermentation *in vitro*. Therefore, the objectives were to investigate 1) OBCFA profiles; 2) fermentation parameters; 3) MCP synthesis and 4) relevant bacterial populations; and then evaluate if the concentrations of OBCFA might be informative of these parameters based on pure carbohydrate incubation *in vitro*.

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 rumen 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 NH_4HCO_3, $NaHCO_3$, Na_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, $C_{12}H_6NO_4Na$, trypticase peptone, $C_3H_7NO_2S \cdot HCl \cdot H_2O$ and $Na_2S \cdot 9H_2O$ 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 black) were taken out of the incubator and placed into ice to end the fermentation. Subsequently, approximate 5 mL of fermented inoculum from each flask was subsampled for the measurements of pH value (Sartorius, pH-Meter PB-10, Goettingen, Germany), NH_3-N and volatile fatty acids (VFA) concentrations. Then another 4 mL of inoculum 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% K_2CO_3 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 N_2 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 Equity^{TM}-1 capillary column (15 m × 0.1 mm × 0.1 μm; Supelco, Inc., Sigma-Aldrich, 28039-U) with N_2 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 NH_3-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 primes 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 = $2^{-(\text{Ct target-Ct total bacteria})}$, 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 $-0.5 > \rho > 0.5$ and $P < 0.05$, where ρ 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 varied 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 cellulolytic bacteria (including *R. albus*, *R. flavefaciens* and *B. fibrisolvens*) and three amylolytic bacteria (including *S. ruminantium*, *R. amylophilus* and *S. bovis*) populations were measured using real-time qPCR system. Among them, *R. flavefacien* (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 cellulolytic 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 iso-C16:0 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 ($P < 0.05$; $\rho = 0.37 \sim 0.82$). 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 cellulolytic 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 significance detected, the correlations with ρ 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, 23.76%; 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-methybutyrate 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 C94), 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 provision 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 ($\rho = 0.21$, $P < 0.05$) whereas positive relationship between *S. bovis* and C15:0 ($\rho = 0.44$, $P < 0.05$) and total production of OBCFA ($\rho = 0.37$, $P < 0.05$) 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_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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None.

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Tables

Table 1
Ingredients and chemical compositions of diets for donor animals

Ingredients (%)		Chemical composition	
Chinese wildrye	43.0	NE_L [2], 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 [1]	0.5		
Total	100.0		

[1]Contained the following per kg of the premix: V_A 8000000 IU, V_D 700000 IU, V_E 10000 IU, Fe 1600 mg, Cu 1500 mg, Zn 10000 mg, Mn 3500 mg, Se 80 mg, I 120 mg, Co 50 mg.

[2]NE_L is calculated value and other nutrient levels are measured values.

Table 2
Primers used for real-time PCR quantification

	Primer sequence (5′-3′)	Product size (bp)	Reference
General bacteria	F:CGGCAACGAGCGCAACCC R:CCATTGTAGCACGTGTGTAGCC	130	Denman and McSweeney (2006)
Ruminococcus albus	F:GTTTTAGGATTGTAAACCTCTGTCTT R:CCTAATATCTACGCATTTCACCGC	273	Yang (2007)
Ruminococcus flavefaciens	F:GATGCCGCGTGGAGGAAGAAG R:CATTTCACCGCTACACCAGGAA	278	Yang (2007)
Butyrivibrio fibrisolvens	F:TAACATGAGTTTGATCCTGGCTC R:CGTTACTCACCCGTCCGC	113	Yang (2007)
Selenomonas ruminantium	F:CAATAAGCATTCCGCCTGGG R:TTCACTCAATGTCAAGCCCTGG	138	Stevenson and Weimer (2007)
Streptococcus bovis	F:TTCCTAGAGATAGGAAGTTTCTTCGG R:ATGATGGCAACTAACAATAGGGGT	127	Stevenson and Weimer (2007)
Ruminobacter amylophilus	F:CTGGGGAGCTGCCTGAAT R: CATCTGAATGCGACTGGTTG	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	iso-C17:0	anteiso-C17:0	C17:0	TC15[1]	TC17[2]	TIFA[3]	TAFA[4]	TOC
F:S = 0:100[8]	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	0.79
	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	1.48
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		0.015	0.034	0.049	0.016	0.018	0.025	0.019	0.080	0.037	0.038	0.045	0.05
P	F:S	< .0001	< .0001	< .00011	< .0001	0.0011	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .00
	Time	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .00
	F:S × Time	0.0003	0.001	< .0001	< .0001	0.13	< .0001	0.0006	< .0001	< .0001	0.01	< .0001	< .00
Polynomial contrast - F:S	Linear	0.11	0.44	< .0001	< .0001	0.02	< .0001	0.0002	< .0001	< .0001	< .0001	< .0001	< .00
	Quadratic	0.37	0.78	< .0001	0.03	0.54	0.0002	0.001	0.001	0.54	0.32	0.02	< .00
Polynomial contrast - Time	Linear	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .00
	Quadratic	0.27	0.0004	< .0001	0.09	0.75	< .0001	< .0001	< .0001	< .0001	0.67	0.95	0.00

[1] TC15 = iso-C15:0 + anteiso-C15:0 + C15:0;[2] TC17 = iso-C17:0 + anteiso-C17:0 + C17:0[3] TIFA = iso-C15:0 + iso-C16:0 + iso-C17:0[4] TAFA = anteiso-C15:0 + ante
C15:0 + C17:0[6] TBCFA = iso-C15:0 + iso-C16:0 + iso-C17:0 + anteiso-C15:0 + anteiso-C17:0[7] OBCFA = iso-C15:0 + anteiso-C15:0 + C15:0 + iso-C16:0 + iso-C17:
[8]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		mg/g DM
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	0.03	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
	Time	< .0001	< .0001	< .0001	0.24	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
	F:S × Time	0.68	< .0001	0.02	0.001	< .0001	0.56	0.007	< .0001	0.99	0.20
Polynomial contrast - F:S	Linear	0.12	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
	Quadratic	0.36	< .0001	< .0001	< .0001	< .0001	0.05	< .0001	0.001	0.12	0.35
Polynomial contrast - Time	Linear	< .0001	< .0001	0.0002	0.38	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
	Quadratic	0.01	< .0001	0.0002	0.17	< .0001	0.004	< .0001	< .0001	0.88	0.90

[1]MCP = microbial crude protein synthesis; [2]F : S = Fiber : Starch.

Table 5

Effect of different ratios of fiber and starch on ruminal bacteria populations[1] in 24 h of incubation *in vitro*

	Time	*R. albus*	*R. flavefaciens*	*B. fibrisolvens*	*S. ruminantium*	*R. amylophilus*	*S. bovis*
	h	10^{-2}%	10^{-2}%	%	%	%	10^{-2}%
F:S = 0:100[2]	6	0.19	3.44	11.38	0.70	14.94	0.19
	12	0.10	0.20	14.78	0.67	14.82	0.09
	18	0.06	0.14	6.76	0.45	24.04	0.25
	24	0.09	0.42	2.23	0.61	9.83	0.25
F:S = 25:75	6	0.40	1.15	17.40	0.97	10.93	0.07
	12	0.03	0.32	4.14	0.52	16.72	0.17
	18	0.07	0.28	5.71	0.42	23.19	0.15
	24	0.07	0.71	3.89	0.45	8.34	0.68
F:S = 50:50	6	0.16	1.85	10.33	0.44	17.58	0.08
	12	0.23	1.58	6.77	0.80	28.91	0.20
	18	0.13	1.44	9.50	0.47	17.01	0.15
	24	0.09	2.12	5.84	0.58	13.00	0.29
F:S = 75:25	6	0.30	1.30	24.48	0.88	11.50	0.19
	12	0.27	3.56	9.74	0.90	17.02	0.47
	18	0.34	13.41	15.36	0.66	4.18	0.45
	24	0.56	7.64	8.48	0.86	1.82	0.40
F:S = 100:0	6	0.17	0.81	29.28	0.48	0.74	0.12
	12	0.22	1.70	9.47	0.70	0.79	0.14
	18	0.70	7.17	10.79	0.96	0.30	0.07
	24	1.48	21.76	8.50	1.11	0.53	0.04
SEM		0.001	0.020	0.023	0.002	0.032	0.001
P	F:S	< .0001	< .0001	< .0001	0.10	< .0001	0.0001
	Time	0.001	< .0001	< .0001	0.614	0.001	0.0004
	F:S × Time	< .0001	< .0001	0.001	0.18	0.02	0.0002
Polynomial	Linear	< .0001	< .0001	< .0001	0.03	< .0001	0.25
contrast - F:S	Quadratic	0.002	0.004	0.07	0.40	< .0001	0.004
Polynomial contrast-Time	Linear	0.002	< .0001	< .0001	0.92	0.02	< .0001
	Quadratic	0.01	0.05	0.01	0.49	0.0003	0.29

[1] Bacteria population was measured as a proportion of the total estimated rumen bacterial 16S rRNA gene (relative quantification = $2^{-(CT-target\ bacteria-CT-total\ bacteria)}$); [2]F : S = Fiber : Starch.

Table 6
Summary statistics of variables utilized for the correlation analysis

	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[1]	2.43	0.88	1.05	4.25
TC17[2]	1.67	0.32	0.99	2.66
TIFA[3]	1.39	0.27	0.75	2.07
TAFA[4]	1.56	0.39	0.73	2.77
TOCFA[5]	1.57	0.62	0.79	3.09
TBCFA[6]	2.95	0.63	1.48	4.64
OBCFA[7]	4.52	1.10	2.30	7.10
Ruminal fermentation parameters and MCP synthesis				
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/propinate	2.08	0.28	1.43	2.59
NH_3-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				
R. albus, 10^{-2}%	0.29	0.36	0.02	2.17
R. flavefaciens, 10^{-2}%	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^{-2}%	0.23	0.21	0.04	1.10

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

Figures

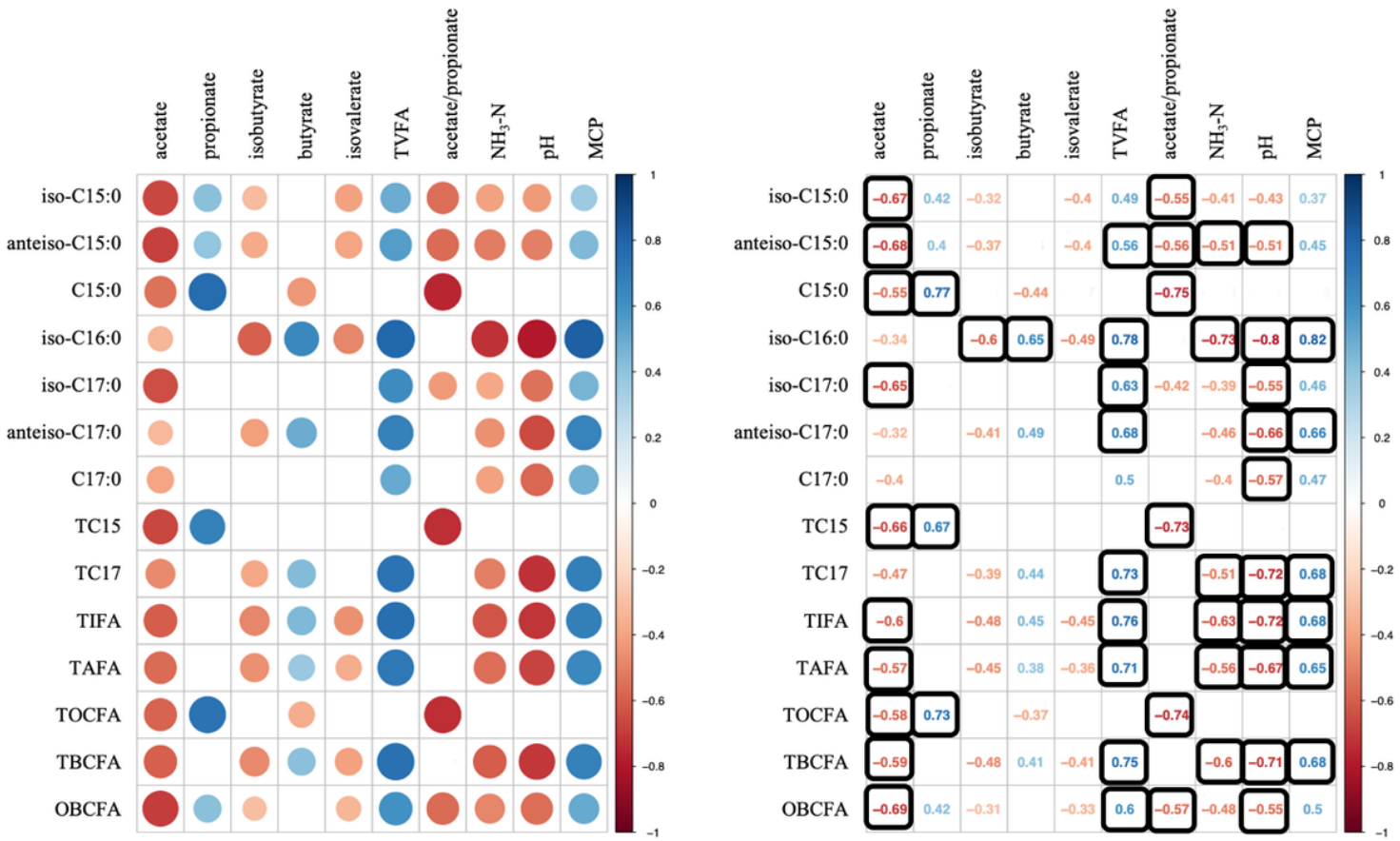

Figure 1

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 $-0.50 > \rho > 0.50$.

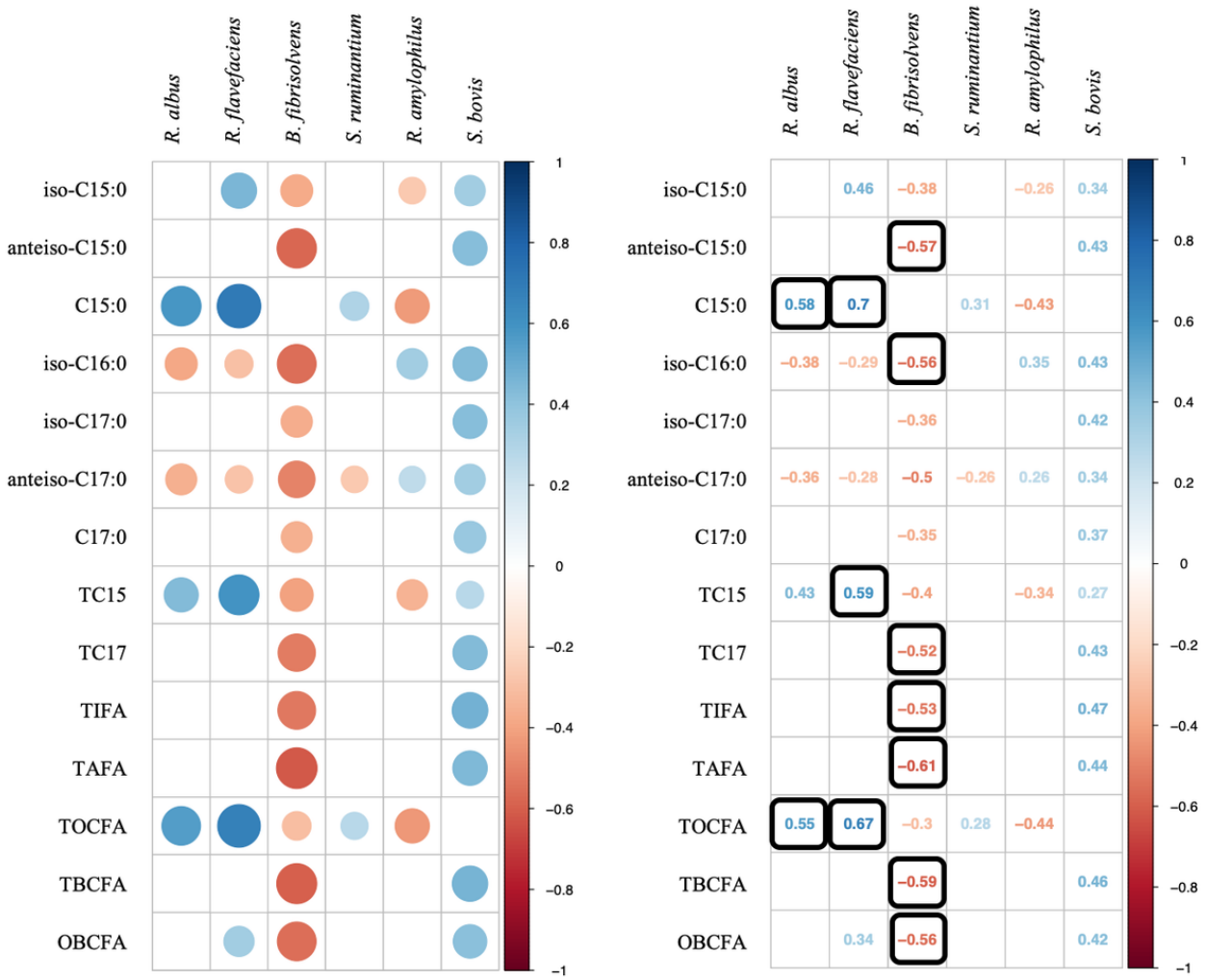

Figure 2

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 $-0.50 > \rho > 0.50$