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Rumen fermentation and acetogen population changes in response to an exogenous acetogen TWA4 strain and *Saccharomyces cerevisiae* fermentation product*

Chun-lei YANG[1], Le-luo GUAN[2], Jian-xin LIU[1], Jia-kun WANG[†‡1]

([1]*Institute of Dairy Science, MoE Key Laboratory of Molecular Animal Nutrition, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China*)

([2]*Department of Agricultural, Food & Nutritional Science, Faculty of Agricultural, Life & Environmental Sciences, University of Alberta, Edmonton, AB, T6G 2P5, Canada*)

[†]E-mail: jiakunwang@zju.edu.cn

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Abstract: The presence of yeast cells could stimulate hydrogen utilization of acetogens and enhance acetogenesis. To understand the roles of acetogens in rumen fermentation, an *in vitro* rumen fermentation experiment was conducted with addition of acetogen strain (TWA4) and/or *Saccharomyces cerevisiae* fermentation product (XP). A 2×2 factorial design with two levels of TWA4 (0 or 2×10^7 cells/ml) and XP (0 or 2 g/L) was performed. Volatile fatty acids (VFAs) were increased ($P<0.05$) in XP and TWA4XP, while methane was increased only in TWA4XP ($P<0.05$). The increase rate of microorganisms with formyltetrahydrofolate synthetase, especially acetogens, was higher than that of methanogens under all treatments. *Lachnospiraceae* was predominant in all acetogen communities, but without close acetyl-CoA synthase (ACS) amino acid sequences from cultured isolates. Low-*Acetitomaculum ruminis*-like ACS was predominant in all acetogen communities, while four unique phylotypes in XP treatment were all amino acid identified low-*Eubacterium limosum*-like acetogens. It differs to XP treatment that more low-*A. ruminis*-like and less low-*E. limosum*-like sequences were identified in TWA4 and TWA4XP treatments. Enhancing acetogenesis by supplementation with an acetogen strain and/or yeast cells may be an approach to mitigate methane, by targeting proper acetogens such as uncultured low-*E. limosum*-like acetogens.

Key words: Acetogen, *Saccharomyces cerevisiae* fermentation product, Rumen fermentation, Methanogen
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1 Introduction

Endogenous rumen microbiota can convert fiber to volatile fatty acid (VFA), and also result in the production of hydrogen and carbon dioxide (Kamra *et al*., 2012). When hydrogen builds up, it inhibits the oxidation of reduced nicotinamide adenine dinucleotide (NADH), leading to the accumulation of lactic acid and reduction of the fermentation (Wolin *et al*., 1997). To maintain a balanced environment for fermentation in the rumen, methanogens can perform methanogenesis, which converts hydrogen and carbon dioxide to methane. However, this process results in 2%–15% loss of feed energy (Johnson and Johnson, 1995) and exacerbation of greenhouse effects.

Recent studies have shown that hydrogen is less used for methanogenesis in the gastrointestinal tract of termites (Breznak, 1994) and Australian marsupials (Ouwerkerk *et al*., 2009) due to the predominant acetogenesis in the gut. Reductive acetogens are a group of bacteria that can produce acetate from hydrogen

‡ Corresponding author
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ORCID: Jia-kun WANG, http://orcid.org/0000-0002-7213-3721
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and carbon dioxides ($4H_2+2CO_2 \rightarrow CH_3COOH+2H_2O$) using the acetyl-CoA pathway (Drake *et al*., 2006). This suggests that acetogens may serve as hydrogen sinks in experiments, which seek to lower methane emissions (van Nevel and Demeyer, 1995; Morvan *et al*., 1996; Joblin, 1999) in cattle. Joblin *et al*. (1999) has reported that methane production decreased by 97% and 64%, respectively, when a *Methanobrevibacter smithii* sp. isolated from a grazing sheep was grown on H_2/CO_2 *in vitro* in the presence of a rumen acetogen isolate. Although an acetogen originating from sewage-sludge, *Peptostreptococcus productus*, competed successfully against methanogens in a simulated gastro-intestinal fermenter (Nollet *et al*., 1997a), such competition failed when *in vitro* ruminal digesta was used (Nollet *et al*., 1997b; 1998). Chaucheyras-Durand *et al*. (1995b) have shown that the presence of yeast cells could stimulate utilization of hydrogen by acetogens and enhance acetogenesis in an experiment utilizing a co-culture of acetogen and methanogen. Therefore, we hypothesized that the methanogenesis in the rumen is reduced by the enhanced acetogenesis with a supplement of *Saccharomyces cerevisiae* fermentation product (XP) and exogenous acetogens.

TWA4, a novel reductive homoacetogen isolated from forestomach contents of female tammar wallabies, could outcompete *M. smithii* in high hydrogen or heterotrophic growth on glycerol (with low hydrogen generated during fermentation) (Gagen *et al*., 2014). Therefore, in this study we assessed the effects of TWA4 and XP on methane production, rumen fermentation, methanogen, and acetogen population, as well as acetogenic diversity using the *in vitro* rumen fermentation system.

2 Materials and methods

2.1 Acetogen and *S. cerevisiae* fermentation product

TWA4 strain was kindly provided by Dr. Chris MCSWEENEY (Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia) and was revived from anaerobic glycerol medium using the modified AC11.1 medium (Gagen *et al*., 2010) with H_2 and CO_2 in a proportion of 1:3 at 120 kPa. After growing three generations, the cell density was counted using a hemocytometer (Strober, 2001). Original XP was supplied by Diamond V (Cedar Rapids, IA, USA).

2.2 Experimental design

The effects of TWA4 and XP supplementation on ruminal fermentation, methane production, methanogen population, abundance, and diversity of rumen acetogens were determined using a 2×2 factorial design using *in vitro* rumen fermentation system. The four treatments were the Control (without TWA4 or XP), TWA4 (2×10^7 cells/ml TWA4 without XP), XP (2 g/L XP without TWA4), and TWA4XP (2×10^7 cells/ml TWA4 with 2 g/L XP).

2.3 *In vitro* rumen fermentation

The rumen fluid was collected from three ruminally fistulated lactating Chinese Holstein cattle (raised in Hangzhou Zhengxing Animal Husbandry Co. Ltd., China) fed twice daily with a mixed diet (roughage:concentrate=55:45) before morning feeding and strained through four layers of gauze into a pre-warmed and insulated bottle. The care and use of fistulated cattle was approved by the Animal Care Committee of Zhejiang University (Hangzhou, China). Rumen fluid was processed under continuous flushing with CO_2. The 120 ml serum bottle containing 0.5 g dry substrates (50:50 (w/w) mixture of Chinese wild rye meal and corn silage), 40 ml buffered medium, and 10 ml rumen fluid was anaerobically incubated at 39 °C using the semi-automated reading pressure technique (Mauricio *et al*., 1999). To get the final concentration of 2 g/L XP for the XP and TWA4XP treatments, 0.1 g XP was added into the serum bottle following the supplement of 0.5 g dry substrates. The 2.5 ml growing TWA4 medium was injected into the bottle for TWA4 and TWA4XP treatments, while 2.5 ml medium without TWA4 inoculation was injected into the control and XP treatments. A pressure transducer, connected with a computer, was used to measure the accumulated head-space gas pressure through the *in vitro* incubation. Gas pressure was recorded after 6, 12, 24, and 48 h of incubation and was subsequently converted to the volumes of gas production (GP). At the same time intervals, 2 ml of head-space gas was collected with an airtight needle (SGE Analytical Science, Australia) to measure the methane production using gas chromatography (GC-2010, Shimadzu, Kyoto, Japan)

equipped with a Flame Ionization detector (Hu *et al*., 2005). For each treatment, triplicate bottles were included and three blanks were included simultaneously to correct the GP values for gas release from endogenous substrates.

At the end of incubation (48 h), 3 ml mixed rumen samples were collected from each bottle and three bottles were sampled under oxygen-free CO_2, and immediately stored at −80 °C to await further determination of the quantity of acetogens and methanogens, the diversity of acetogens, and the measurement of VFAs. VFAs were determined using the procedure described by Hu *et al*. (2005). Dry matter degradation (DMD) was measured using the modified nylon bag method (Goering and van Soest, 1970).

2.4 Total DNA extraction and real-time quantitative polymerase chain reaction (PCR)

Total DNA was extracted from rumen fluid collected from a 48-h incubation period using the bead-beating method as previously reported (Gagen *et al*., 2010). The primers specific to formyltetrahydrofolate synthetase gene (*fhs*) and methyl coenzyme-M reductase A (*mcrA*) genes (Table 1) were used to enumerate microorganisms with formyltetrahydrofolate synthetase **(**FTHFS) (Xu *et al*., 2009) and methanogens (Denman *et al*., 2007), respectively. The 16S rRNA gene of total bacteria was amplified with the primers as reported by Denman and McSweeney (2006) as shown in Table 1, and the copy number of total bacterial 16S rRNA gene was used as the reference to calculate the relative quantification of target. Real-time PCR was performed with SYBR green in ABI 7500 using the program: one cycle of initial denaturation at 95 °C for 10 s, 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min. The relative abundance of each marker gene was estimated as: relative quantification of target=$2^{-(C_{\text{T target}}-C_{\text{T total bacteria}})}$.

2.5 Acetyl-CoA synthase gene sequencing

To investigate the effect of TWA4 and XP on acetogen diversity, acetyl-CoA synthase (ACS) genes were amplified and sequenced from the triplicate of DNA extracted from a 48-h incubation period as previously described (Gagen *et al*., 2010). The amplicon triplicates from each treatment were then pooled on an equal concentration basis (Checked by Qubit 2.0, Invitrogen, USA) for clone library construction using the pGEM-T Easy® vector (Promega Co., Madison, Wisconsin, USA) and *Escherichia coli* competent cell DH5α (TaKaRa, Dalian, China) according to manufacturer's instructions. In total, 96, 96, 94, and 92 clones were randomly sequenced from the control, TWA4, XP, and TWA4XP libraries, respectively, and were then sequenced with T7 primer (Beijing Genomic Institute, China). The Primer Premier 5.0 (PREMIER Biosoft International, CA, USA) was used to translate DNA sequences to amino acid sequences before alignment. Sequence similarity to ACS was determined by BLASTP analysis (Gish and States, 1993) using all existing bacterial ACS amino acid sequences in NCBI database. The ACS amino acid sequences were grouped into phylotypes at a distance of 0.01 using Jones-Taylor-Thornton Matrix of MEGA6 (Tamura *et al*., 2013), because *Blautia schinkii* and *Ruminococcus obeum* shared 96.3% ACS amino acid identity, while 0.03 and 0.02 distance levels could not be clustered in our ACS amino acid sequences. The richness of ACS amino acid sequences was evaluated by the number of phylotypes and Chao1 index. The evenness was analyzed by Simpon's diversity and Pielou's evenness indices, calculated from Shannon's diversity index (Felsenstein, 1993). Relative abundance of the phylotypes was determined as the sequence numbers in the phylotypes/total number of sequences. The Chao1,

Table 1 Primer sets used to amplify genes encoding total bacteria 16S rRNA, FTHFS, *mcrA*, and ACS in this study

Target	Direction	Primer sequence (5'→3')	Reference
Total bacteria	F	CGGCAACGAGCGCAACCC	Denman and McSweeney, 2006
	R	CCATTGTAGCACGTGTGTAGCC	
FTHFS	F	GTWTGGGCWAARGGYGGMGAAGG	Xu *et al*., 2009
	R	GARGAYGGWTTTGAYATYAC	
mcrA	F	TTCGGTGGATCDCARAGRGC	Denman *et al*., 2007
	R	GBARGTCGWAWCCGTAGAATCC	
ACS	F	CTBTGYGGDGCIGTIWSMTGG	Gagen *et al*., 2010
	R	AARCAWCCRCADGADGTCATIGG	

F: forward; R: reverse; FTHFS: formyltetrahydrofolate synthetase; *mcrA*: methyl coenzyme-M reductase A; ACS: acetyl-CoA synthase

Simpon's and Shannon's diversity indices, Good's coverage ($1-n_{scp}/n_{total}$, n_{scp} is the number of single clone phylotypes and n_{total} is the total number of sequences) were estimated using the summary single command in MOTHUR (Schloss *et al*., 2009). Unifrac.weighted in MOTHUR was used to compare the structure of acetogen community based on ACS amino acid sequences, and then to run the principal coordinates analysis (PCoA) with the pcoa command in MOTHUR. Bootstrapped neighbour joining tree of deduced ACS amino acid sequences was constructed with MEGA 6.06 with 100 resamplings. Similar amino acid sequences identified by BLASTP analysis and ACS of TWA4 (AEL12814 and AEL12815) and *Methanococcoides methylutens* (KGK98586) were selected as references for tree construction. Putative ACS amino acid sequences determined in the present study have been submitted to the GenBank database under accession numbers KR152340 to KR152636.

2.6 Statistical analyses

Data for DMD, methane production, VFA, and abundance of acetogens and methanogens were analyzed by two-way analysis of variance in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) with individual bottles as the experimental unit, TWA4 and XP supplementation as main effects, where the TWA4×XP interaction was significant, and a secondary test was conducted to separate the efficacy of TWA4 within XP (Robinson *et al*., 2006). Multiple comparisons means among treatments were completed by Duncan's multiple range tests. Significance was declared if $P<0.05$.

3 Results

3.1 Rumen fermentation characteristics

3.1.1 Methane production

The methane production (ml/g substrate) was not affected ($P>0.05$) by TWA4 or XP alone except for TWA4 at 6 h incubation (Table 2). When supplemented with TWA4XP, methane production was increased ($P<0.05$) at all time points by 20% to 107% compared with the control.

3.1.2 Fermentation parameters

DMD was not affected ($P>0.05$) by treatments (Table 2). XP increased ($P<0.05$) total VFA and acetate and butyrate concentrations and TWA4XP increased ($P<0.05$) acetate, propionate, butyrate, and total VFA concentrations compared with the control. TWA4 showed no effect ($P>0.05$) on VFA production.

Table 2 Effects of acetogen, *S. cerevisiae* fermentation product, co-addition of acetogen and yeast culture on methane emission and rumen fermentation parameters

Parameter	Control	TWA4	XP	TWA4XP	SEM	*P*-value						
						TWA4×XP	TWA4			XP		
							Main	+XP	−XP	Main	+TWA4	−TWA4
CH_4 (ml/g substrate)												
6 h	7.76±0.15^{c}	9.89±0.71^{b}	8.20±0.18^{c}	16.06±0.42^{a}	0.43	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.48
12 h	27.69±0.91^{b}	28.30±2.85^{b}	24.76±0.20^{b}	38.36±0.10^{a}	1.50	<0.01	<0.01	<0.01	0.78	<0.05	<0.01	0.20
24 h	53.97±0.75^{b}	48.55±0.22^{b}	49.58±2.88^{b}	64.76±1.46^{a}	1.66	<0.01	<0.05	<0.01	0.05	<0.01	<0.01	0.10
48 h	60.19±0.20^{b}	59.88±0.61^{b}	59.23±0.83^{b}	88.26±1.03^{a}	0.74	<0.01	<0.01	<0.01	0.77	<0.01	<0.01	0.38
CH_4 (% of GP)												
6 h	17.12±0.17^{c}	20.76±0.003^{b}	17.70±1.47^{c}	27.20±0.73^{a}	0.77	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	0.59
12 h	31.44±0.75^{ab}	31.40±2.93^{ab}	29.12±0.28^{b}	35.35±0.04^{a}	1.52	0.07	0.08			0.61		
24 h	40.25±0.57^{ab}	36.11±0.27^{c}	38.30±1.74^{bc}	39.84±1.03^{ab}	1.03	<0.05	0.24	0.32	<0.05	0.42	<0.05	0.22
48 h	35.51±0.19^{b}	35.57±0.31^{b}	36.00±0.22^{b}	43.74±0.47^{a}	0.32	<0.01	<0.01	<0.01	0.90	<0.01	<0.01	0.31
VFA (mmol/L)												
Total	99.46±0.15^{c}	100.05±0.49^{c}	103.16±0.76^{b}	114.94±1.08^{a}	0.71	<0.01	<0.01	<0.01	0.58	<0.01	<0.01	<0.01
Acetate (A)	61.40±0.30^{c}	62.15±0.17^{c}	64.28±0.41^{b}	70.82±1.85^{a}	0.60	<0.01	<0.01	<0.01	0.39	<0.01	<0.01	<0.01
Propionate (P)	23.11±0.15^{b}	23.14±0.28^{b}	23.63±0.30^{b}	27.18±0.23^{a}	0.25	<0.01	<0.01	<0.01	0.95	<0.01	<0.01	0.18
Butyrate	9.46±0.09^{c}	9.47±0.08^{c}	10.04±0.15^{b}	11.07±0.28^{a}	0.17	<0.05	<0.05	<0.01	0.96	<0.01	<0.01	<0.05
A:P*	2.66±0.03^{ab}	2.69±0.03^{ab}	2.72±0.03^{a}	2.61±0.03^{b}	0.028	<0.05	0.17	<0.05	0.46	0.76	0.07	0.14
DMD (%)	62.34±0.26	61.39±2.33	59.88±2.42	62.59±1.67	1.88	0.65	0.74			0.36		

GP: gas production; VFA: volatile fatty acids; DMD: dry matter degradation; TWA4: acetogen; XP: *Saccharomyces cerevisiae* fermentation product; TWA4XP: co-addition of acetogen and yeast culture; SEM: pooled standard error of the mean (n=3). For the *P*-value, ×: interaction; +: with supplementation; −: without supplementation. Data, shown as mean±standard error (n=3), within a row with different superscripts differ significantly ($P<0.05$). * Data were expressed as proportions

3.2 FTHFS and methanogen abundance

After a 48-h incubation period, the relative abundances of FTHFS and methanogens were increased (P<0.05) with the addition of TWA4, XP, and TWA4XP, though relative abundance of FTHFS was much lower than methanogen abundance (Table 3). The increase in the abundance of FTHFS was greater than that of methanogens (P<0.05) for XP and TWA4XP compared with TWA4 (Table 3). The increases of FTHFS and methanogens were 382.1% and 209.4% for TWA4, 656.0% and 478.1% for XP, and 679.1% and 421.9% for TWA4XP, respectively, compared with the control.

3.3 Acetogen diversity

In total, 73, 70, 74, and 80 ACS amino acid sequences were detected in the control, TWA4, XP, and TWA4XP groups, respectively. Further amino acid similarity (99%) analysis identified 15 phylotypes for the control, 10 phylotypes for TWA4, 12 phylotypes for XP, and 7 phylotypes for TWA4XP with 3 common phylotypes among the four treatments (Table 4). Coverage analysis and a rarefaction curve indicated that the sequences obtained covered the majority of acetogen communities in all treatments with 89.0% for the control, 94.3% for TWA4, 93.2% for XP, and 95.0% for TWA4XP groups, respectively (Table 4, Fig. 1). From the results of numbers of phylotypes and Chao1, the richness of deduced ACS amino acid sequences was highest in the control group, followed by XP, TWA4, and TWA4XP. The control and XP had the highest Pielou's evenness followed by TWA4, while TWA4XP had the highest Simpson indices value.

Eight out of 22 of the phylotypes showed high (98%–100%) similarity to putative ACS amino acid of uncultured bacterium at GenBank uploaded by Gagen *et al*. (2010). All these phylotype sequences showed 62%–90% similarity to the nearest valid taxa including *Acetitomaculum ruminis*, *R.* sp. CAG:9, *R. obeum*, *Eubacterium limosum*, *B. schinkii*, *B. hydrogenotrophica*, and *B. wexlerae* (Table 5). Only 3 phylotypes were common phylotypes among the four treatments (Fig. 2), which occupied about 79.1% of the total ACS sequences. Phylotypes 1 and 2 had 86%

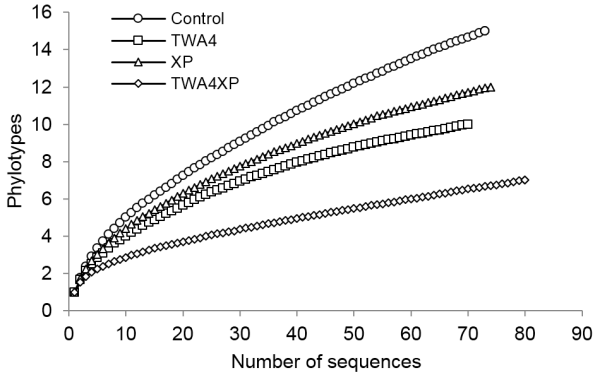

Fig. 1 Rarefaction curve of observed phylotypes of acetyl-CoA synthase generated at 99% identity cutoff value

Table 3 Least squares means of log ratio to total bacterial 16S rDNA of FTHFS and methanogen population after 48 h incubation

Parameter	log ratio to total bacterial 16S rDNA					*P*-value						
	Control	TWA4	XP	TWA4XP	SEM	TWA4×XP	TWA4			XP		
							Main	+XP	−XP	Main	+TWA4	−TWA4
FTHFS	−6.88±0.035^c	−6.19±0.023^b	−6.19±0.023^a	−5.98±0.019^a	0.026	<0.01	<0.01	0.72	<0.01	<0.01	<0.01	<0.01
Methanogen	−2.57±0.165^c	−2.01±0.020^b	−1.73±0.037^a	−1.78±0.022^a	0.086	<0.01	<0.05	0.73	<0.01	<0.01	0.09	<0.01

FTHFS: formyltetrahydrofolate synthetase; TWA4: acetogen; XP: *Saccharomyces cerevisiae* fermentation product; TWA4XP: co-addition of acetogen and yeast culture; SEM: pooled standard error of the mean (n=3). For the P-value, ×: interaction; +: with supplementation; −: without supplementation. Data, shown as mean±standard error (n=3), within a row with different superscripts differ significantly (P<0.05)

Table 4 Acetogen community diversity indices of four treatments at 99% cutoff

Treatment	No. of OTUs	Chao1	Shannon	Pielou's evenness	Simpson	Good's coverage
Control	15	22.0	1.89	0.61	0.23	89.0
TWA4	10	13.0	1.48	0.58	0.32	94.3
XP	12	14.5	1.62	0.61	0.30	93.2
TWA4XP	7	13.0	0.96	0.37	0.51	95.0

OTUs: operational taxonomic units. Good's coverage ($1-n_{scp}/n_{total}$, n_{scp} is the number of single clone phylotypes and n_{total} is the total number of sequences) was estimated using the summary.single command of MOTHUR. The Pielou's evenness was estimated as Shannon/ln(Chao1). The total number of sequences was 73, 70, 74, and 80 in the control, TWA4, XP, and TWA4XP treatments, respectively

Fig. 2 Phylogenetic analysis of deduced acetyl-CoA synthase amino acid sequences

P is the phylotypes generated at 99% identity cutoff value. GenBank accession numbers of reference sequences are shown after the species names. Bootstap values of ≥50% are shown at the nodes. The low-*A. ruminis*-like sequences and low-*E. limosum*-like sequences are marked with solid circles (●) and squares (■), respectively

Table 5 Phylotype distribution of acetyl-CoA synthase between individual treatments

Phylotype	Total Clones	Control	TWA4	XP	TWA4XP	Nearest valid taxa		Nearest relative	
						Description	Identity (%)	Description	Identity (%)
1	158	30	35	38	55	*A. ruminis*, ADR32215	86	Uncultured bacterium, ADR32256	96
2	62	15	19	11	17	*A. ruminis*, ADR32215	86	Uncultured bacterium, ADR32274	98
3	15	10	3	2	0	*A. ruminis*, ADR32215	90	Uncultured bacterium, ADR32250	98
4	15	4	4	3	4	*B. schinkii*, ADR32225	76	*B. schinkii*, ADR32225	76
5	11	0	0	11	0	*E. limosum*, ADR32216	70	Uncultured bacterium, ADR32288	96
6	4	1	3	0	0	*B. hydrogenotrophica*, ADR32222	84	Uncultured bacterium, ADR32235	90
7	4	2	0	2	0	*E. limosum*, ADR32216	72	Uncultured bacterium, ADR32278	100
8	4	1	2	0	1	*E. limosum*, ADR32216	62	Uncultured bacterium, AEM54104	100
9	3	2	1	0	0	*B. hydrogenotrophica*, ADR32222	79	Uncultured bacterium, ADR32267	82
10	3	1	1	1	0	*E. limosum*, ADR32216	65	Firmicutes bacterium CAG:124, WP_021932507	73
11	3	2	1	0	0	*E. limosum*, ADR32216	68	Firmicutes bacterium CAG:170, WP_022223268	76
12	3	1	0	1	1	*B. wexlerae*, WP_026648192	77	Uncultured bacterium, ADR32238	98
13	3	1	0	2	0	*R. obeum*, WP_044925744	73	Uncultured bacterium, ADR32247	100
14	1	0	0	0	1	*B. hydrogenotrophica*, ADR32222	66	Uncultured bacterium, AEM54114	100
15	1	0	0	1	0	*E. limosum*, ADR32216	69	Uncultured bacterium, ADR32278	98
16	1	1	0	0	0	*E. limosum*, ADR32216	68	Firmicutes bacterium CAG:124, WP_021932507	73
17	1	0	0	1	0	*R.* sp. CAG:9, WP_022380765	83	*R.* sp. CAG:9, WP_022380765	83
18	1	0	0	0	1	*A. ruminis*, ADR32219	73	Firmicutes bacterium CAG:170, WP_022223268	79
19	1	0	1	0	0	*A. ruminis*, ADR32219	70	Firmicutes bacterium CAG:170, WP_022223268	76
20	1	1	0	0	0	*B. wexlerae*, WP_029676805	80	Uncultured bacterium, ADR32267	86
21	1	0	0	1	0	*E. limosum*, ADR32216	73	*E. limosum*, ADR32216	73
22	1	1	0	0	0	*E. limosum*, ADR32216	69	Uncultured bacterium, ADR32288	81
Total	297	73	70	74	80				

similarity with *A. ruminis*, 96% and 98% similarities with uncultured bacterium. Phylotype 4 had 76% similarity with *B. schinkii* (Table 5).

Based on amino acid identified phylotypes, uncultured bacteria sequences were predominant in all acetogen communities. The dominant acetogens were unchanged in the treatments (phylotypes 1 and 2; Table 5 and Fig. 3) while the acetogen communities were significantly different among four treatments (weighted significance <0.001; Fig. 3b), whose community changes were reflected in the appearance new phylotypes. For XP treatment, about all the four unique phylotypes were identified as low-*E. limosum*-like acetogens (Table 5). There were no TWA4 ACS amino acid sequences actually recovered in the TWA4 and TWA4XP libraries (Fig. 2).

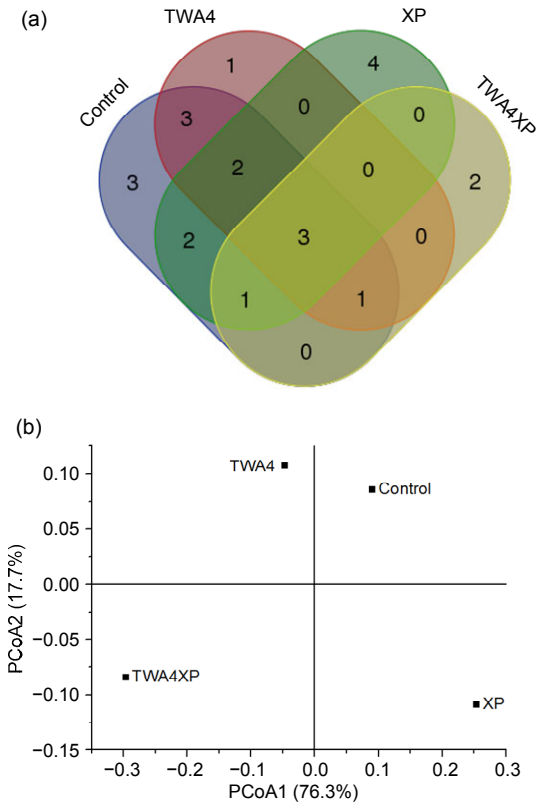

Fig. 3 Unique and common acetyl-CoA synthase phylotypes identified in the four treatments (a), and the principal coordinates analysis (PCoA) for the four treatments (b)

4 Discussion

Ruminal methane production through enteric fermentation is of global concern due to its contribution to greenhouse gas emission as well as accounting for dietary energy loss in animals. Since the 1950s much effort has been expended trying to reduce methane emission from ruminants. Although the use of agents including chemicals and antibiotics has been successful, their use has been limited due to the probability of high quantities of residuals remaining in the animal products and feces (Kobayashi, 2010; Patra, 2012). Methanogenesis is the primary method of facilitating the consumption of hydrogen in the rumen, followed by fumarate reduction (Asanuma and Hino, 2000; Mitsumori and Sun, 2008), sulfate reduction (Morvan *et al*., 1996; Sahakian *et al*., 2010), nitrate and nitrite reduction (Sakthivel *et al*., 2012), and reductive acetogenesis (Henderson *et al*., 2010). Evidence from *in vitro* incubations of rumen contents (Nollet *et al*., 1997a; le Van *et al*., 1998) and methanogen-free lambs (Fonty *et al*., 2007) indicates that acetogens can function as hydrogenotrophs to suppress methanogenesis. Thus, enhancing reductive acetogenesis in the rumen may be an effective strategy to mitigate methane.

Lopez *et al*. (1999) investigated the ability of six reductive acetogens to prevent the accumulation of methane *in vitro* and found that only two of them, *E. limosum* strains ATCC 8486 and Ser 5, decreased methane production by about 5% after a 24-h incubation period, while total VFA was not affected ($P>0.05$) by the addition of the six acetogens. As with Lopez's study, the addition of TWA4 alone did not change the methane and VFA production in our experiment. The TWA4, XP, and TWA4XP failed to improve the DMD, revealing that no additional hydrogen and nutrients were produced from the substrates in these treatments compared with the control.

Yeast, as a feed additive, has been confirmed as being able to provide nutrients and vitamins to microorganisms (Chaucheyras-Durand *et al*., 1995a). Chaucheyras-Durand and Fonty (2001) reported that the supplementation of *S. cerevisiae* CNCM I-1077 tended to improve the *in sacco* degradation of wheat straw, and increased VFA production. From that study, Lascano and Heinrichs (2009) reported *S. cerevisiae* yeast culture increased rumen VFA concentration without influencing DMD. They concluded that the increased total VFA concentration with unchanged molar proportions of individual VFA was the result of the increased fermentation rate created by yeast culture supplement. However, reasons for inconsistency between the increased fermentation rate and unchanged DMD were not explained. As with the above study, our results showed that DMD remained unchanged, suggesting that the increased VFA by XP addition or XP and TWA4 co-addition was not a product of fermentable organic matter, such as cellulose or starch.

Chaucheyras-Durand *et al*. (1995b) showed that the presence of yeast cells stimulated the utilization of hydrogen by acetogens and enhanced acetogenesis, in a co-culture of acetogens and methanogens. In our XP and TWA4XP treatments, the increased VFA was mainly due to the acetate (Table 2). With the *fhs* primers of Xu *et al*. (2009), the rate of acetogen increase was higher than that of methanogens in XP and

TWA4XP, suggesting that XP treatments may alter the acetogen communities and increase acetogenesis. However, according to Gagen *et al*. (2010), the *fhs* primers used to enumerate acetogens in our experiment could recover partial FTHFS sequence from a wider range of rumen acetogens, although multiple spurious amplicons could be generated from some acetogens and rumen microbial DNA. Until now, no appropriate real-time PCR primers were reported for acetogens, it could only deduce that XP treatments may increase the FTHFS biochemical pathway of rumen microorganisms, especially acetogens, which would cause an increase in VFA production.

The diversity of the acetogen community in our experiment showed that *Lachnospiraceae* was the dominant acetogen in the rumen fermentation system, but without close sequences from cultured isolates, which was similar to the results of Gagen *et al*. (2010). However, the species richness of our experiment was lower than that of Gagen *et al*. (2010). This difference may be due to the production accumulation *in vitro* system. It is possible that TWA4 could not survival after a 48-h fermentation period; however, the acetogen community was significantly changed by adding TWA4 alone or with XP, and therefore we posit that future studies are needed to track the fate of TWA4 with the change of fermentation time.

Acetogen phylotypes were increased by the addition of XP (Table 5 and Fig. 3a) with four unique phylotypes where amino acid was identified as low-*E. limosum*-like acetogen (Table 5). *E. limosum* and *A. ruminis* were all isolates from rumen by Sharak Genthner *et al*. (1981) and Greening and Leedle (1989), respectively. These bacteria can utilize H_2/CO_2, one-carbon compounds, to produce acetate. However, in contrast to *A. ruminis*, *E. limosum* can utilize amino acid to produce acetate and butyrate, and its growth can be stimulated by amino acids (Sharak Genthner *et al*., 1981; Pacaud *et al*., 1985). Amino acids were a substrate for methanogen growth and methanogensis (Mathrani and Boone, 1985; Chaucheyras-Durand *et al*., 1995b) as well. Therefore, enhancing acetogenesis by supplement with acetogen strain and/or yeast cells may be a way of mitigating methane, targeting acetogens such as *E. limosum* which utilize substrates such as amino acids to facilitate growth and acetogenesis. In order to further support our speculation, future experiments should next try isolating the low-*E. limosum*-like acetogens to test their nutritional characteristics and competition with methanogens.

5 Conclusions

The efficacy of adding TWA4 alone in the *in vitro* rumen fermentation system was limited, while with the substrate provided by XP, TWA4 could enhance acetogenesis by changing the acetogen community to low-*A. ruminis*-like acetogens. However, methanogenesis in rumen was enhanced by the co-addition. The XP addition could enhance acetogenesis with unchanged methanogenesis by changing the acetogen community to low-*E. limosum*-like acetogens, suggesting that enhancing acetogenesis by supplementation with acetogen strain and/or yeast cells may be a way to mitigate methane, with proper targeted acetogens such as the uncultured low-*E. limosum*-like acetogens.

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Compliance with ethics guidelines

Chun-lei YANG, Le-luo GUAN, Jian-xin LIU, and Jia-kun WANG declare that they have no conflict of interest.

All management and experimental procedures were approved by the Animal Care Committee of Zhejiang University, China and were carried out in accordance with the University's guidelines for animal research.

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中文概要

题　目：外源添加产乙酸菌和酿酒酵母发酵物对瘤胃发酵特性及产乙酸菌菌群结构的影响

目　的：酵母可以促进产乙酸菌利用氢生成乙酸。为了解产乙酸菌在瘤胃发酵中的作用，本试验研究了产乙酸菌单独添加及其与酿酒酵母发酵物联合添加对瘤胃发酵特性及产乙酸菌菌群结构的影响。

创新点：通过瘤胃甲烷生成量与产乙酸菌菌群结构关联，揭示降低瘤胃甲烷生成的新途径。

方　法：试验采用 2×2 双因素试验设计，产乙酸菌 TWA4 的添加量为 0 和 2×10^7 cells/ml，酿酒酵母发酵物 XP 的添加量为 0 和 2 g/L。

结　论：瘤胃体外发酵研究发现，单独添加 XP 以及 TWA4 和 XP 联合添加可增加挥发性脂肪酸产量（$P<0.05$），TWA4 和 XP 联合添加显著增加了甲烷生成量（$P<0.05$）。单独添加 TWA4、XP 以及两者联合添加均增加了含有甲酰四氢叶酸合成酶的微生物的数量，尤其是产乙酸菌的数量，这类微生物的增长幅度高于产甲烷菌。各处理产乙酸菌均以 *Lachnospiraceae* 为主，但与已培养的微生物的相似性较低。与 *Acetitomaculum ruminis* 低相似性的一类未知的产乙酸菌在各处理中均占优势，但 XP 的添加增加了与 *Eubacterium limosum* 低相似未知产乙酸菌的数量。与 XP 不同，TWA4 及其与 XP 的联合添加使与 *A. ruminis* 低相似性的一类未知的产乙酸菌的数量增加，与 *E. limosum* 低相似未知产乙酸菌的数量降低。因此，添加与 *E. limosum* 低相似的未知产乙酸菌和（或）酵母可能是降低瘤胃甲烷生成的一种有效途径。

关键词：产乙酸菌；酿酒酵母发酵物；瘤胃发酵；产甲烷菌