Microbial diversity and volatile profile of traditional fermented yak milk

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ABSTRACT

Previous research reported that fermented yak milk had a diverse microbial composition. For this study, raw yak milk, qula, and fermented yak milk samples were collected from the Aba Tibetan autonomous region of China. The genus and species microbial composition of these samples were analyzed by high-throughput sequencing of 16S rRNA and *groEL* gene amplicons, and the volatile profile of the samples was quantified by gas chromatography–mass spectrometry. The results indicated variation in abundance of microbiota at the genus level among the fermented yak milk samples, with *Lactobacillus* as the most abundant genus in the majority of samples, ranging from 41.6 to 98.3%. The volatile profile of the samples varied among those collected from different villages. Correlations between bacterial composition and volatile compounds of the samples were also observed. *Lactobacillus* displayed a significant correlation with volatile compounds such as benzaldehyde, 2,3-pentanedione, ethanol, and ethyl acetate, whereas the samples with relatively high abundance of *Streptococcus* and *Lactococcus* displayed relatively low contents of volatile compounds.

Key words: fermented yak milk, microbial diversity, volatile profile

INTRODUCTION

The yak (*Bos grunniens*) is a long-haired bovid found throughout the Himalaya region of southern Central Asia, the Tibetan Plateau, Mongolia, and Russia, with over 95% of the world's total yak population currently

in China (Zhang et al., 2008). Fermented yak milk is a traditional dairy product in northwestern China that is generally prepared using qula—a traditional product made by defatting, acidifying, and air-drying yak milk (Duan et al., 2008; Yang et al., 2018) and fermenting at 4 to 15°C for 12 to 36 h—as the starter culture.

The diverse microbial composition of fermented yak milk has been reported previously (Bai et al., 2010; Bao et al., 2012; Liu et al., 2012; Liu et al., 2015). At the genus level, *Firmicutes* are dominated by *Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Leuconostoc*, whereas *Ascomycota* were mainly *Saccharomycodes*. At the species level, *Lactobacillus* species, including *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* were detected, with the presence of *Streptococcus thermophilus* and *Lactococcus lactis* in certain samples as well. Although revealing, previous research mainly focused on identification of the microbiota composition; further analysis beyond the relative abundance of individual genera is desired.

Although the 16S rRNA profiling methods have been widely used to investigate microbial flora at phylum and genus level, its limitation at species level has been reported, and genetic markers such as the *groEL* gene have been developed for identification of lactic acid bacteria in various samples (Bergonzelli et al., 2006; Blaiotta et al., 2008; Shevtsov et al., 2011; Hu et al., 2017; Milani et al., 2018). In previous studies we have developed effective approaches for the analysis of *Bifidobacterium* and *Lactobacillus* in fecal and naturally fermented samples using the *groEL* gene (Hu et al., 2017; Xie et al., 2019). Thus, a more detailed analysis of microbial diversity of traditionally fermented yak milk samples would provide useful information for screening and application of lactic acid bacteria.

In addition, few studies have investigated the volatile profile of traditional fermented yak milk (Chi et al.,

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Table 1. Sampling information for different yak milk products from 15 families in 5 villages in Ruorgai, the Aba Tibetan autonomous region of China¹

Family code	Raw yak milk (M)	Qula (Q)	Fermented yak milk (T)	Sampling village
A1	MA1	QA1	TA1	Dagenggou
A2	MA2	QA2	TA2	Dagenggou
A3	—	QA3	—	Dagenggou
A4	MA4	QA4	TA4	Dagenggou
B1	MB1	QB1	TB1	Axi
B2	—	QB2	—	Axi
C1	—	QC1	TC1	Duoma
C2	MC2	QC2	TC2	Duoma
D1	—	QD1	TD1	Banyou
D2	MD2	QD2	TD2	Banyou
D3	MD3	QD3	TD3	Banyou
D4	MD4	QD4	TD4	Banyou
D5	—	QD5	TD5	Banyou
E1	—	QE1	TE1	Xiangdong
E2	—	QE2	TE2	Xiangdong

¹Fermented yak milk was made from raw yak milk, with qula as the starter culture, by individual families.

2017). Therefore, in the present study, the objective was to evaluate the microbial diversity of yak milk, qula, and fermented yak milk from Ruorgai in the Aba Tibetan autonomous region of China. Furthermore, the variation in abundance at genus level of lactic acid bacteria and at species level of *Lactobacillus* among the fermented yak milk samples were evaluated, as well as the volatile profile of the fermented yak milk products.

MATERIALS AND METHODS

Sampling Region

Samples were collected from Ruorgai, the Aba Tibetan autonomous region of China, located at 32°56' to 34°19' N and 102°08' to 103°39' E. It has a subarctic climate, with an elevation of approximately 3,500 m above sea level, average precipitation of 656.8 mm per year, and annual average temperature of 1.1°C. During the sampling period, the temperature ranged from 4 to 15°C.

Sampling

A total of 36 samples, including 8 yak milk, 15 qula, and 13 fermented yak milk, were collected from 15 families across 5 villages of Ruorgai, the Aba Tibetan autonomous region of China, including Dagenggou, Axi, Douma, Banyou, and Xiangdong, from June 5 to 10, 2018. Information about the samples is shown in Table 1. Samples with the same sampling code are from the same family, and fermented yak milk was made by individual families from raw yak milk with qula as the starter culture. During the sampling, 30 to 50 mL of raw milk and fermented milk, and around 30 g of qula were collected in triplicate from each household, packaged in

50-mL sterile plastic bottles, and frozen immediately. After sampling the samples were transferred on ice to the laboratory within 24 h.

Physical and Chemical Analysis

Proximate Composition. The total solids content was determined by drying 5 g of each sample at 110°C for 7 h in a capsule according to AOAC method 990.20. The determination of fat contents of the samples was carried out gravimetrically, according to AOAC method 2000.18, using petroleum ether as solvent for Soxhlet extraction. The protein content was measured using a Kjeltac 8400 (Foss Tecator, Hoganas, Sweden) according to AOAC method 991.23. Lactose was determined according to AOAC method 896.01.

pH Determination and Titratable Acidity. Samples' pH was measured using a digital 320 pH meter (Mettler Toledo, Zurich, Switzerland). Ten grams of each sample was diluted with 70 mL of distilled water and mixed thoroughly for pH measurement. The acidity of the sample was determined by titration, following the method described by the AOAC (947.05) using phenolphthalein (Riedel-de Haën AG, Seelze, Germany) as an indicator.

High-Throughput Sequencing of 16S rRNA Amplicons

Total bacterial DNA was extracted from each sample using the FastDNA SPIN kit for soil and the Fast-Prep Instrument (Qbiogene Inc., Carlsbad, CA) according to the manufacturer's instructions. The extracted DNA was examined on agarose gel and quantified using the Quant-iT™ HS ds-DNA assay kit (Invitrogen, Paisley,

UK) in combination with the QuBit™ fluorometer. The bacterial V3-V4 16S rRNA region was amplified with the primer pairs 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The amplification procedure were performed according to previous research (Jia et al., 2016). The *groEL* gene of the *Lactobacillus* genus was amplified with the primer pairs 308F (5'-GCYGGTGCWAACCCNGTTGG-3') and 806R (5'-AANGTNCCVCGVATCTTGTT-3'), and the amplification procedure was conducted according to previous protocols developed in our laboratory (Hu et al., 2017). The PCR products were multiplexed in a single pool in equimolar amounts, based on the QuBit quantification data. The PCR product pool was then purified using the solid-phase reverse immobilization method of the Quant-iT PicoGreen dsDNA assay kit (Qiagen, Hilden, Germany) and sequenced at FASTERIS SA (Geneva, Switzerland). The TurSeq DNA LT sample preparation kit (Illumina Inc., San Diego, CA) was used for amplicon library preparation, and the sequencing reaction was performed using a MiSeq Illumina instrument (Illumina Inc.) with V3 chemistry, generating 300-bp paired-end reads. The bioinformatic sequence analysis was conducted as described by Mao et al. (2015).

Volatile Compounds Analysis

The volatile compounds of fermented yak milk were detected and quantified by headspace solid-phase micro-extraction GC/MS. Six milliliters of each sample was kept in 20-mL clear glass screw top vials at room temperature for 10 min before analysis. Each sample was added with decanoic acid ethyl ester (0.05 mg/mL; Sigma-Aldrich, Milwaukee, WI) as an internal standard. The selection of DVB/CAR/PDMS fiber (divinylbenzene/carboxen/polydimethylsiloxane; Supelco, Bellefonte, PA), column and GC program were based on the references, with modification (Hettinga et al., 2008; Settachaimongkon et al., 2014; Dan et al., 2017). After equilibration of the samples for 30 min at 50°C, the fiber was exposed to the headspace for 5 min at 50°C. A Trace1300 GC fitted with a single-quadrupole ISQ MS (Thermo Fisher Scientific Inc., Waltham, MA,) and a Rtx-Wax capillary column (30 m, 0.25-mm internal diameter, 0.25-mm thickness). Desorption was performed within 2 min in splitless mode at 240°C. The injection temperature was 240°C, and the transfer line and ion source were at 240°C. The mass detector was operated in electron impact mode at a voltage of 70 eV, and mass spectra were recorded with a mass range of 33 to 500 *m/z* with 0.2 scans/s. Helium was used as the carrier gas at a constant linear velocity of 15.0 mL/min. The temperature program was the following:

30°C (3 min), from 30 to 225°C at 15°C/min, held for 5 min at 225°C. The NIST 2011 standard mass spectral database was used to identify volatile compounds based on retention time and mass-spectral similarity match (more than 85%). Authentic standards were applied to confirm the marker volatile compounds of the assignments. All analysis was conducted in triplicate.

Statistical Analysis

Data on the compositions of yak milk and fermented yak milk samples were analyzed for statistical significance via ANOVA using the statistical package SPSS 21.0 (SPSS Inc., Chicago, IL). Principal component analysis, partial least squares discrimination analysis, and hierarchical clustering analysis were performed using SIMCA 14.1 (Umetrics AB, Umea, Sweden). Differences between samples were analyzed using Tukey's test and considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Proximate Composition, pH, and Titratable Acidity

The proximate composition (total solids, fat, protein, lactose), pH, and titratable acidity of yak milk (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-16753>) were similar to the results of previous studies of yak milk (He et al., 2011). The mean total solids content, protein, lactose content, and fat content of the yak milk were $15.10 \pm 0.90\%$ (SD), $4.12 \pm 0.54\%$, $4.71 \pm 0.83\%$, and $5.69 \pm 0.88\%$, respectively. The mean titratable acidity of yak milk was $23.04 \pm 1.31^\circ\text{T}$, and the pH value was in the range of 6.36 to 6.62.

Variation in the composition of fermented yak milk samples was observed (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-16753>) and expected for naturally fermented dairy products considering factors such as raw milk, starter culture, and fermentation conditions. The mean total solids, fat, and lactose contents of the fermented yak milk samples were $14.78 \pm 1.47\%$, $5.25 \pm 1.02\%$, and $2.13 \pm 0.71\%$, close to the compositions reported in a previous study of naturally fermented yak milk (Zhang et al., 2008). Protein content and titratable acidity of our samples were $2.80 \pm 0.31\%$ and $107.40 \pm 29.09^\circ\text{T}$, lower than previously reported values of 5.37% and 158°T (Zhang et al., 2008).

Microbial Diversity Analysis

The Simpson_1-D, Chao-1, and Shannon_H indices are shown in Table 2; these indices are used to characterize the diversity and richness of each sample. For the 3 types of collected samples, the highest levels of

diversity and richness were observed in yak milk (mean Simpson_1-D = 0.73, mean Chao-1 = 600.5), followed by qula (mean Simpson_1-D = 0.49, mean Chao-1 = 362.6) and fermented yak milk (mean Simpson_1-D = 0.32, mean Chao-1 = 238.6), which illustrated that the diversity and richness of microbiota in the samples decreased significantly during fermentation. The high microbial diversity and richness of yak milk was probably due to the milking and exposure of the raw milk to the environment before fermentation. As a starter culture, qula was usually air-dried after fermentation; the decrease of pH value and water activity might partly inhibit excessive microbial growth, as reported in fermented meat products (Fontana et al., 2016). For fermented yak milk, preparation, including heating of raw yak milk and addition of qula as starter culture, and overnight fermentation all contributed to the lower diversity and richness compared with raw milk and qula, as also observed previously by Bao et al. (2012).

As shown in the heat map, *Firmicutes* was the most abundant phylum (Supplemental Figure S1A; <https://doi.org/10.3168/jds.2019-16753>), and *Lactobacillus*, *Lactococcus*, and *Streptococcus* were the most abundant genera in the samples (Supplemental Figure S1B; <https://doi.org/10.3168/jds.2019-16753>). *Firmicutes* dominated in all of the samples, and similar results have been observed in naturally fermented foods, including fermented dairy products and fermented llama meat sausages (Hunt et al., 1997), probably due to the ability of lactic acid bacteria to produce acid and antibacterial substances, which help them to outcompete other microbial populations during fermentation.

At the genus level, Pearson correlation analysis was performed on the bacterial composition (Figure 1). The correlation coefficients of the genus compositions of yak milk and qula ranged from -0.2 to 0.9 (with a mean value of 0.26), whereas the coefficients of the genus compositions of qula and fermented yak milk were in the range 0.3 to 0.82 (with a mean value of 0.56). The results indicated that the genus compositions of qula and fermented yak milk were more similar than the genus compositions of yak milk and fermented yak milk, which might be attributed to the fact that qula is typically added as the starter culture during preparation of fermented yak milk.

Bacterial Composition of Fermented Yak Milk

The relative bacterial abundances at phylum, genus, and *Lactobacillus* species levels of fermented yak milk are shown in Figure 2. The taxonomical assignment showed that 87% of the sequences were classified at phylum level, 92% at genus level, and 99% at *Lacto-*

Table 2. α -Diversity values of lactic acid bacteria in samples of yak milk, qula, and fermented yak milk from Ruorgai, the Aba Tibetan autonomous region of China

Sampling code ¹	Simpson_1-D	Shannon_H	Chao-1
MA1	0.91	3.61	541.50
MA2	0.62	1.77	245.40
MA4	0.53	1.37	545.50
MB1	0.30	0.86	184.40
MC2	0.88	3.37	1,060.00
MD2	0.81	2.33	500.50
MD3	0.93	3.54	753.50
MD5	0.84	3.31	973.00
QA1	0.71	1.69	363.00
QA2	0.43	1.16	416.30
QA4	0.61	1.27	331.10
QB2	0.68	1.71	393.20
QC1	0.15	0.48	452.40
QC2	0.56	1.25	222.50
QD1	0.44	0.99	241.20
QD2	0.50	1.27	349.40
QD4	0.57	1.26	354.90
QD5	0.54	1.32	251.00
QE1	0.22	0.62	614.00
TA1	0.80	2.50	568.00
TA2	0.48	0.87	106.00
TA3	0.48	0.90	221.10
TA4	0.30	0.62	329.10
TB1	0.16	0.39	335.30
TB2	0.11	0.30	201.50
TC1	0.69	1.48	306.60
TC2	0.26	0.59	192.60
TD1	0.18	0.51	110.60
TD2	0.70	1.58	430.90
TD3	0.39	0.69	70.67
TD4	0.04	0.15	133.00
TD5	0.05	0.17	73.00
TE1	0.07	0.20	249.10
TE2	0.13	0.33	251.70

¹Each sampling code comprises an indicator of the type of product (M = raw yak milk, Q = qula, T = fermented yak milk) and an individual family code. Detailed information on the sampling codes is shown in Table 1.

bacillus species level. At phylum level, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* displayed relatively high abundance (Figure 2A), with the abundance of the other phyla, such as *Actinobacteria*, *Tenericutes*, and *Verrucomicrobia*, lower than 0.06% (data not shown). *Firmicutes* accounted for more than 99% at phylum level in 13 of the 15 fermented yak milk samples (TA2, TA3, TA4, TB1, TB2, TC1, TC2, TD1, TD3, TD4, TD5, TE1, and TE2), with 87.67% in TA1 and 91.35% in TD2. A similar trend of dominant *Firmicutes* was observed in previous studies on fermented yak milk (Zhang et al., 2008; Chen et al., 2009).

At genus level (Figure 2B), *Lactobacillus* was observed as the most abundant genus in 9 samples: TA1 (41.60%), TA2 (68.67%), TA3 (70.74%), TA4 (84.66%), TC1 (67.57%), TC2 (94.57%), TD1 (94.38%), TD3 (74.44%), and TD5 (98.26%). *Streptococcus* predominated in 4 samples, TB1 (91.21%), TD4 (98.09%),

TE1 (96.57%), and TE2 (92.99%), with *Lactococcus* predominating in TB2 (94.43%) and TD2 (46.75%). We also recorded 16 other genera, such as *Acetobacter*, *Bifidobacterium*, and *Aggregatibacter*, with abundances lower than 0.8% (data not shown).

The results of the bacterial community at the *Lactobacillus* species level are shown in Figure 2C. The *Lactobacillus* species *L. delbrueckii* was the most abundant in TA1 (99.39%), TB1 (98.74%), TD3 (99.38%), TE1 (99.08%), and TE2 (98.56%), whereas *L. helveticus* was the most abundant species in TA4 (99.33%), TC1 (99.67%), TC2 (96.05%), and TD2 (83.38%). The results illustrated that generally only one *Lactobacillus* species was dominant in each sample of the collected fermented yak milk. Besides *L. delbrueckii* and *L. helveticus*, 5 other *Lactobacillus* species, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *L. fermentum*, *L. plantarum*, and *Lactobacillus amylovorus* were detected with abundances lower than 0.02% (data not shown).

Volatile Compound Analysis

Fourteen volatile compounds were identified in the fermented yak milk samples, including 6 acids, 4 ketones, 1 aldehyde, 1 alcohol, 1 ester, and 1 ether (Table 3). Compared with commercial fermented bovine milk samples (data not shown), the fermented yak milk samples generally displayed higher dimethyl disulfide and ethanol content, probably due to differences in microbial composition and fermentation conditions. We should also point out that the volatile profile is highly dependent on the selection of GC column and analytical conditions. In the present study a polar column was chosen, based on the fact that fermented yak milk has high fat content; a more nonpolar column will result in different volatile profile, with more nonpolar volatile compounds.

Principal component analysis of the volatile compounds is shown in Figure 3A, and the first 2 compo-

Figure 1. Inter-correlation analysis of bacterial diversity of raw yak milk, qula, and fermented yak milk samples. Pearson coefficient was calculated, and correlation coefficients (Corr) are shown in different colors. Each sampling code (left and bottom edges of the figure) comprises an indicator of the type of product (M = raw yak milk, Q = qula, T = fermented yak milk) and an individual family code. Detailed information on the sampling codes is shown in Table 1.

nents contributed to 63.7% of the variation. Samples from villages C, A, B, D, and E are distributed on the horizontal axis from right to left in the figure. The

content of acetic acid in the samples collected from village C ranged from 98.08 to 177.27 $\mu\text{g/kg}$, significantly higher than those of villages D (7.85 to 91.3 $\mu\text{g/kg}$)

Figure 2. Relative abundance of bacteria in fermented yak milk (A) at phylum level, (B) at genus level (relative abundance >1%), and (C) at species level within the genus *Lactobacillus*. Each sampling code (bottom edge of each panel) comprises an indicator of the type of product (M = raw yak milk, Q = qula, T = fermented yak milk) and an individual family code. Detailed information on the sampling codes is shown in Table 1.

Table 3. Volatile compound contents ($\mu\text{g/kg}$) of samples of fermented yak milk from Ruergai, the Aba Tibetan autonomous region of China¹

Sampling code ²	Acetic acid	Hexanoic acid	Octanoic acid	Nonanoic acid	Decanoic acid	Benzoic acid	Acetone	2,3-Butanedione	2,3-Pentanedione	Acetoin	Benzaldehyde	Ethanol	Ethyl acetate	Dimethyl disulfide	Total
TA1	10.11 $\pm 3.14^a$	8.75 $\pm 1.89^{ab}$	2.95 $\pm 0.28^{abc}$	6.88 $\pm 2.87^{abc}$	ND	186.06 $\pm 74.58^{bc}$	6.54 $\pm 0.93^{abcd}$	8.84 $\pm 0.26^{de}$	3.45 $\pm 0.35^d$	25.31 $\pm 2.22^{cd}$	8.39 $\pm 2.05^{bcd}$	21.67 $\pm 0.74^{ab}$	4.69 ^c $\pm 0.65^b$	3.12 $\pm 0.76^{abcd}$	296.76
TA2	25.50 $\pm 1.67^a$	14.65 $\pm 1.76^{abc}$	6.24 $\pm 1.25^{cd}$	10.98 $\pm 2.83^{abc}$	ND	25.59 $\pm 0.40^a$	7.84 $\pm 5.37^{cd}$	8.29 $\pm 2.57^{cde}$	3.03 $\pm 0.75^{cd}$	23.27 $\pm 1.23^{bcd}$	8.01 $\pm 0.63^{bcd}$	7.44 $\pm 0.88^{ab}$	2.05 $\pm 0.13^{ab}$	4.84 $\pm 0.04^{cd}$	147.73
TA3	22.13 $\pm 0.02^a$	22.78 $\pm 2.24^{bc}$	8.87 $\pm 1.82^{de}$	8.86 $\pm 3.59^{abc}$	10.17 $\pm 1.82^c$	26.33 $\pm 4.40^a$	6.41 $\pm 1.20^{abcd}$	2.75 $\pm 0.69^{ab}$	ND	15.54 $\pm 2.84^{abc}$	11.46 $\pm 0.83^{de}$	598.61 $\pm 28.40^f$	5.45 $\pm 0.02^c$	3.03 $\pm 0.66^{abcd}$	742.32
TA4	186.55 $\pm 45.11^d$	7.87 $\pm 1.40^{ab}$	2.88 $\pm 0.20^{abc}$	4.50 $\pm 1.48^{abc}$	5.09 $\pm 1.88^b$	47.45 $\pm 1.94^a$	4.42 $\pm 0.96^{abcd}$	11.40 $\pm 0.04^{ef}$	ND	107.43 $\pm 11.34^f$	9.74 $\pm 2.09^{cd}$	125.19 $\pm 1.20^d$	13.53 $\pm 2.27^d$	1.90 $\pm 0.29^{abc}$	527.95
TB1	11.41 $\pm 3.57^a$	2.37 $\pm 0.91^a$	0.80 $\pm 0.30^{ab}$	ND	1.94 $\pm 0.20^{ab}$	16.24 $\pm 4.24^a$	2.31 $\pm 0.67^{abcd}$	3.34 $\pm 1.31^{ab}$	2.03 $\pm 1.11^{bcd}$	14.3 $\pm 4.67^{abc}$	3.07 $\pm 0.66^{ab}$	35.12 $\pm 9.60^{ab}$	0.43 $\pm 0.22^a$	1.76 $\pm 0.82^{abc}$	95.12
TB2	24.90 $\pm 0.68^a$	12.48 $\pm 1.80^{abc}$	4.16 $\pm 0.40^{bc}$	6.00 $\pm 2.30^{abc}$	20.32 $\pm 2.94^d$	46.64 $\pm 9.41^a$	8.19 $\pm 1.59^d$	10.87 $\pm 1.16^e$	ND	17.86 $\pm 4.27^{abc}$	5.38 $\pm 1.24^{abc}$	44.67 $\pm 1.51^{abc}$	ND	2.49 $\pm 0.11^{abc}$	203.96
TC1	98.08 $\pm 6.95^c$	24.88 $\pm 1.96^c$	10.92 $\pm 0.56^e$	12.57 $\pm 1.70^{bc}$	ND	101.57 $\pm 32.20^{abc}$	5.94 $\pm 1.32^{abcd}$	15.99 $\pm 1.22^f$	2.12 $\pm 0.26^{bcd}$	54.86 $\pm 1.23^e$	5.91 $\pm 0.27^{abc}$	272.94 $\pm 13.10^e$	5.26 $\pm 0.41^c$	4.54 $\pm 1.89^{bcd}$	615.49
TC2	177.27 $\pm 16.81^d$	23.94 $\pm 0.32^c$	5.07 $\pm 1.38^{cd}$	14.91 $\pm 0.29^c$	30.25 $\pm 1.20^e$	219.13 $\pm 5.50^c$	8.03 $\pm 0.82^d$	12.63 $\pm 0.59^{ef}$	3.75 $\pm 0.21^d$	37.54 $\pm 7.74^{de}$	15.28 $\pm 0.02^e$	93.39 $\pm 3.89^{cd}$	ND	6.13 $\pm 0.95^d$	647.32
TD1	42.22 $\pm 8.31^{ab}$	5.13 $\pm 1.61^a$	1.15 $\pm 0.48^{ab}$	1.57 $\pm 0.05^{ab}$	ND	36.92 $\pm 16.18^a$	1.88 $\pm 1.07^{abcd}$	2.49 $\pm 0.68^{ab}$	1.43 $\pm 0.16^{abc}$	7.22 $\pm 1.68^{ab}$	2.13 $\pm 0.26^a$	35.09 $\pm 8.18^{ab}$	1.10 $\pm 0.37^a$	0.83 ^a ± 0.16	139.16
TD2	91.30 $\pm 3.43^{bc}$	2.48 $\pm 0.31^a$	ND	ND	ND	13.80 $\pm 1.45^a$	0.84 $\pm 0.05^{ab}$	ND	ND	1.97 $\pm 0.09^a$	2.35 $\pm 0.65^a$	25.39 $\pm 1.81^{ab}$	1.33 $\pm 0.00^a$	1.10 $\pm 0.06^a$	140.56
TD3	25.34 $\pm 16.15^a$	16.19 $\pm 13.30^{abc}$	4.22 $\pm 2.22^{bc}$	ND	ND	101.58 $\pm 83.10^{abc}$	ND	ND	2.12 $\pm 0.86^{bcd}$	11.26 $\pm 6.35^{abc}$	8.02 $\pm 3.00^{bcd}$	54.64 $\pm 33.36^{bc}$	2.35 $\pm 1.40^{abc}$	2.46 $\pm 1.74^{abc}$	228.18
TD4	8.65 $\pm 1.50^a$	3.40 $\pm 0.42^a$	ND	13.05 $\pm 10.28^{bc}$	ND	17.51 $\pm 0.86^a$	7.12 $\pm 1.65^{bcd}$	3.97 $\pm 0.19^{abc}$	2.02 $\pm 0.46^{bcd}$	10.89 $\pm 0.38^{abc}$	5.34 $\pm 1.67^{abc}$	ND	2.47 $\pm 1.06^{abc}$	0.85 $\pm 0.15^a$	75.27
TD5	7.85 $\pm 0.31^a$	10.75 $\pm 1.86^{abc}$	2.57 $\pm 1.17^{abc}$	ND	12.86 $\pm 0.90^c$	67.29 $\pm 3.85^{ab}$	2.75 $\pm 0.68^{abcd}$	ND	ND	0.85 $\pm 0.07^a$	6.59 $\pm 0.16^{abcd}$	0.72 $\pm 0.40^a$	ND	2.56 $\pm 0.18^{abc}$	114.79
TE1	4.32 $\pm 0.75^a$	3.44 $\pm 0.98^a$	1.02 $\pm 0.25^{ab}$	ND	ND	15.69 $\pm 2.09^a$	1.27 $\pm 0.10^{abc}$	3.25 $\pm 0.70^{ab}$	0.39 $\pm 0.13^{ab}$	5.03 $\pm 1.38^a$	1.91 $\pm 0.48^a$	0.45 $\pm 0.06^a$	ND	1.03 $\pm 0.46^a$	37.8
TE2	10.39 $\pm 2.78^a$	7.38 $\pm 2.18^a$	1.14 $\pm 0.22^{ab}$	ND	ND	27.20 $\pm 5.44^a$	2.25 $\pm 0.09^{abcd}$	5.36 $\pm 2.86^{bcd}$	1.13 $\pm 0.51^{ab}$	8.44 $\pm 2.61^{abc}$	4.73 $\pm 1.92^{abc}$	0.68 $\pm 0.12^a$	ND	1.48 $\pm 0.10^{ab}$	70.18

^{a-f}Means in the same row with different superscript letters are significantly different ($P < 0.05$).¹Data presented as means $\pm$ SD. ND = not detected.²Each sampling code comprises an indicator of the type of product (M = raw yak milk, Q = qula, T = fermented yak milk) and an individual family code. Detailed information on the sampling codes is shown in Table 1.

Figure 3. Statistical analysis of volatile flavor compounds in fermented yak milk (T). Each sampling code (TA1–TE2) indicates the individual family who produced the fermented milk; see Table 1 for detailed information. (A) Principal component analysis (PCA); different symbol shapes represent samples from different villages. (B) Hierarchical clustering analysis (HCA), performed according to the scores resulting from PCA. (C) Partial least squares discrimination analysis (PLS-DA). *Lactobacillus* high (black pentagons) or low (open diamonds) abundance represent samples with relative abundance of *Lactobacillus* greater or less than 40% at genus level, respectively. (D) HCA performed according to the scores of PLS-DA.

and E (4.32 to 10.39 $\mu\text{g/kg}$). The samples from village C also displayed the highest content of ethanol, ranging from 93.39 to 272.94 $\mu\text{g/kg}$. In general, samples from village E contained the lowest concentrations of volatile compounds and showed no presence of nonanoic acid, decanoic acid, or ethyl acetate.

The hierarchical clustering analysis of the scores of principle component analysis (Figure 3B) showed that the fermented yak milk samples were divided into 2 groups. The first cluster contained all the samples from villages A and C; the second cluster, all those from villages D and E. However, the 2 samples from village B did not cluster together. It appeared that the volatile profiles of fermented yak milk samples were different among the samples collected from different villages.

Partial least squares discrimination analysis of the volatile compounds in fermented yak milk is shown in Figure 3C, with *Lactobacillus* abundance differentiating around 40%. According to the variable importance for the projection (VIP), the differential compounds within the volatile profile (VIP > 1) included 2,3-butanedione, hexanoic acid, benzaldehyde, octanoic acid,

acetone, disulfide dimethyl, benzoic acid, nonanoic acid, and ethyl acetate. According to the scores of partial least squares discrimination analysis (Figure 3D), the hierarchical clustering analysis indicated that all the samples were divided into 2 clusters. The samples in cluster 2 had lower total content of volatile compounds than those in cluster 1, ranging from 37.8 to 203.96 $\mu\text{g/kg}$ in cluster 2 and 228.18 to 742.32 $\mu\text{g/kg}$ in cluster 1. The *Streptococcus*-predominant and *Lactococcus*-predominant samples all grouped in cluster 2. On the other hand, variation was observed for the *Lactobacillus*-predominant samples, which spread into 2 clusters. Nevertheless, all the samples in cluster 1 were *Lactobacillus*-predominant in bacterial composition at the genus level. It has been demonstrated that many factors affect the generation of volatile compounds during fermentation, such as microbial composition, raw materials, and fermentation conditions (Cheng, 2010; Settachaimongkon et al., 2014). Thus, further analysis is desired for potential correlation between volatile profile and microbial composition of the fermented yak milk samples.

Figure 4. Correlation analysis of lactic acid bacteria genus composition and content of volatile flavor compounds. Pearson coefficients were calculated and considered to be significant when $P < 0.05$. Significant correlated relationships are shown in the figure.

Correlation Analysis Between Volatile Profile and Lactic Acid Bacteria at Genus Level

The correlation analysis of lactic acid bacteria composition of the fermented yak milk samples indicated a negative correlation between the relative abundance of *Lactobacillus* with *Streptococcus* and *Lactococcus* in the present study (Figure 4). Previous research on cross-feeding interactions in *Streptococcus* and *Lactobacillus* showed that the 2 strains could exchange precursors, cofactors, long-chain fatty acids, and other compounds that are associated with biosynthesis or metabolism reactions, and promoted one another's growth (Partanen et al., 2001; Sybesma et al., 2003). However, research has also revealed that the interaction of co-occurring microorganisms is complicated, and cross-feeding interactions can change into competition relationships among microorganisms over time during fermentation. When the proteolytic activity of *L. delbrueckii* was insufficient to meet the demands for branched-chain amino acids and sulfur amino acids by both *Streptococcus thermophilus* and *L. delbrueckii*, competition was observed in the mixed culture (Sieuwerds et al., 2010). The negative correlation between the relative abundance of *Lactobacillus* and *Streptococcus* in the fermented yak milk samples seems to indicate competitive behavior between the 2 bacteria after extended fermentation of yak milk for 12 to 36 h, as observed in the collected samples.

Correlations among the bacterial genera of the fermented milk samples and their content of volatile compounds (Figure 4) implies that the relative abundance of *Lactobacillus* in fermented yak milk had significant positively correlation with the contents of most of the detected volatile compounds, except acetone and butanedione. In contrast, the relative abundance of *Streptococcus* had significant positive correlation with the content of 2,3-pentanedione and ethyl acetate, and significant negative correlation with the other compounds except 2,3-butanedione. The relative abundance of *Lactococcus* had significant positive correlation only with the content of acetic acid.

The generation of volatile compounds in fermented milk, including lactic acid, acetaldehyde, diacetyl, acetoin, and ethanol, involves complex biochemical reactions among carbohydrates, proteins, and lipids (Cheng, 2010; De Prisco et al., 2017). The genetic and metabolic diversity of lactic acid bacteria at the genus and strain levels has been well documented and is responsible for flavor diversification in fermented products (Singh et al., 2007). Therefore, thorough characterization of microbiota profiles is required in order to correlate the variations in flavor profiles observed in the present study. We must also point out that a vari-

ety of other factors besides lactic acid bacteria might contribute to the volatile profiles of the fermented milk, including autolytic microorganisms, fungi, fermentation conditions, and more.

CONCLUSIONS

Variation in abundance of bacteria at the genus level was recorded among fermented yak milk samples. *Lactobacillus* was the most abundant genus in 9 out of 15 fermented yak milk samples. For the rest of the samples, *Streptococcus* predominated in 4, and *Lactococcus* predominated in 2. The volatile profiles of fermented yak milk samples were different among samples collected from different villages. Correlations between bacterial composition and volatile compounds of the traditional fermented yak milk samples were observed. The factors that contributed to the flavor profile of the fermented milk are complicated, and further research needs to be done to reveal the correlation between microbial ecology and flavor profiles of fermented yak milk.

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