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# Fermentation Process of Mulberry Juice-Whey Based Tibetan Kefir Beverage Production

*Bo LI[1,2], Xin GAO[1,2], Na LI[1,2] and Jun MEI[1,2,3]**

*[1]Department of Food Quality and Safety and [2]Institute of Urban Food Safety; Shanghai Urban Construction Vocational College, Shanghai, China; [3]Department of Food Science and Technology, Shanghai Ocean University, Shanghai, China*

**Corresponding author: delightmay@hotmail.com*

**Abstract**

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Mixture of mulberry juice and whey was evaluated as a potential substrate for the production of a beverage by Tibetan kefir grains. Different mulberry juice addition was used. Acidity, pH, volatile flavour compounds as well as microbial communities were determined during 40 h of fermentation at 18°C. Gas chromatography-mass spectroscopy (GC-MS) analysis revealed that ethanol and 3-methyl-1-butanol were dominant alcohols, and ethyl caprylate, ethyl caprate, ethyl acetate and ethyl caproate were the most dominant ester compounds. The microbial communities of fermented beverage were close to kefir grains indicating that they had similar microbial communities gradually during the fermentation process. *Lactococcus* was frequently detected at the beginning and then *Lactobacillus* rapidly proliferated after acclimatizing to the fermentation environment. *Acetobacter* was steadily increasing during the fermentation process. For the fungi, *Candida* was frequently detected with the highest abundances in almost all samples.

**Keywords**: flavour; microbial diversity; mulberry juice; Tibetan kefir; beverage; whey

Kefir grains can be added to cow, goat or sheep milk to produce lactic acid, acetic acid, $CO_2$, alcohol and aromatic compounds (Rosa *et al*. 2017) and they can also be applied to ferment different substrates including cheese whey, fruit juice and molasses (Nouska *et al*. 2015). Black mulberry (*Morus nigra* L.) is one of the most important species of the genus *Morus*, with fruits having substantial levels of total phenolics, total flavonoids, and ascorbic acid (Qin *et al*. 2010). The ripe mulberry fruit is dark red to dark purple, and this colouring arises from the presence of anthocyanins that can prevent oxidation reactions (Eo *et al.* 2014). However, mulberry has a thin skin that is unfavourable for storage or transportation. Postharvest mulberry fruits can be processed into mulberry juice, fermented beverage, jams, vinegar, and wine to prolong its shelf life.

The aim of this study was to evaluate the use of mulberry juice and whey as raw materials to design a probiotic beverage using Tibetan kefir grains as starter culture. Volatile flavour compounds and microbial communities of the novel beverage were determined.

## MATERIAL AND METHODS

***Preparation of fermented beverage***. The whey was mixed with mulberry juice concentrate to 15 °Brix and homogenized at 20 MPa (Ah-basic; ATS Engineering Inc., Canada). Then the mixture were subjected to pasteurization at 63°C for 30 min to inactivate the naturally existing bacterial population followed by cooling at 4°C for 4–5 h until use.

Kefir grains were inoculated into the prepared mulberry juice-whey mixture at 5% (w/v). The beverage was incubated at 18°C for 40 hours. The fermentation runs were assessed every 10 h in order to determine

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the characteristics of beverages. After completion of fermentation the kefir grains were removed and the beverage was kept at a suitable temperature for future analysis.

***Titratable activity and pH values***. Titratable activity (TA) was determined according to Comunian *et al*. (2017) during fermentation (0, 10, 20 30 and 40 h, respectively), the pH values of the kefir samples were measured with an FE 20 pH metre (Mettler-Toledo, USA) at room temperature.

***Determination of volatile flavour compounds***. The volatile flavour compounds were determined by the methods of Nambou *et al*. (2014) and Mei *et al*. (2015). Five grams of mulberry juice-whey beverage (MJWB) samples were used. The volatile compounds were identified by comparison with commercial reference compounds provided by Sigma-Aldrich (St. Louis, USA) and by comparison of their mass spectra with those contained in the NIST 2011.

***Microbial communities***. The genomic DNA was extracted from the MJWB at different fermentation time using the protocol reported by Qu *et al*. (2016), and the V4 region of 16S rRNA gene was amplified using the primer set 520F (5'- GCACCTAAYTGGGYDTAAAGNG-3') and 802R (5'-TACNVGGGTATCTAATCC-3'). PCR amplification was carried out based on the methods described previously (Gu *et al*. 2017), and the resulting PCR amplicons were used for sequencing on the Illumina MiSeq platform (Personalbio, China).

After sequencing, the data processing was conducted as described previously (Qu *et al*. 2016; Gu *et al*. 2017). Heat map was generated using HemI (Heatmap Illustrator, version 1.0) (Deng *et al*. 2014).

## RESULTS AND DISCUSSION

***Changes in TA and pH***. The TA and pH values of MJWB samples during the fermentation process were increased from 65.67°C at 0 h to 156.67°C at 40 h (data not shown). Similarly, pH values for beverage samples were 5.35 at 0 h and they significantly decreased to 4.26 after 40 hours.

***Volatile flavour compounds***. Table 1 show the changes in concentrations of main volatile compounds (area units, AU × $10^7$) present in the samples.

Thirty alcohol compounds were detected including primary, secondary, branched-chain, unsaturated and aromatics alcohols. The total amount of alcohols decreased because of esterification with acids to produce esters. Ethanol and 3-methyl-1-butanol were the most abundant alcohols. The high concentration of ethanol in the beverage is probably due to the high population of yeasts and heterofermentative lactic acid bacteria in the kefir grains, resulting in the formation of high amounts of ethyl esters (Table 1).

Interestingly, aldehydes were present at low levels compared to other aroma groups. Aldehydes are transitory compounds and do not accumulate significantly in the fermentation process because they are rapidly reduced to primary alcohols or oxidized to the corresponding acids.

Ketones have low perception thresholds and are well-known contributors to the aroma. Most of the ketones in the beverage were methyl ketones (Table 1). The most prevalent ketones found in the beverage were acetoin, 2-heptanone, and 2-nonanone. Ketones are abundant constituents in most dairy products, and they have typical smells. 2-nonanone is associated with fruity, floral and musty notes or medicinal/sour notes. Fruity, floral and musty notes are associated with various methyl ketones such as 2-octanone, 2-nonanone, and 2-undecanone, so the presence of these volatile compounds can be considered positive for the beverage flavour.

Acids were present in a considerable number of volatile compounds found in the beverage. They were all short- and medium-chain (C4:0–C12:0) carboxylic acids and lipolysis could be the main pathway responsible for the release of carboxylic acids in the beverage. These carboxylic acids were almost all below their odour thresholds, and so they may not have had an impact on the sensory profile. However, they could play a role as precursors of ethyl esters which made a valuable contribution to the beverage flavour (Dongmo *et al*. 2017). Acetic acid was the representative carboxylic acid, giving a mild to strong sharp vinegar flavour.

In this study, most esters showed statistically significant increases in concentration from 0 h to 30 h, reaching the highest concentration at 30 h and then it decreased. Ethyl esters were the most abundant esters. Although concentrations of some ethyl esters were lower in comparison with other compounds, they have a low detection threshold, and they contribute to fruity and floral notes. The C4-C10 ethyl esters of organic acids, ethyl esters of straight-chain fatty acids and acetates of higher alcohols are widely, if not exclusively, responsible for the fruity aroma of beverages (Sumby *et al*. 2010).

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Table 1. Volatile compounds concentration changes (AU × $10^7$) isolated from mulberry juice-whey beverages during fermentation process

| Compounds | Odour | Concentration changes | R.I. | References |
|---|---|---|---|---|
| Ethanol | alcoholic | 276.44 | 928.4 | Feng *et al.* (2015) |
| 1-Propanol | alcoholic | 2.91 | 1042.4 | Xiao *et al.* (2017) |
| Isobutyl alcohol | | 9.4 | 1112.1 | |
| 1-Butanol | medicinal, phenolic | 1.45 | 1157.4 | Sánchezpalomo *et al.* (2010) |
| 2-Methyl-1-butanol | alcoholic, nail polish | 16.64 | 1212.5 | Xiao *et al.* (2017) |
| 3-Methyl-1-butanol | whiskey, malt | 133.04 | 1214.2 | Xiao *et al.* (2017) |
| 1-Pentanol | green | 0.32 | 1253.9 | March *et al.* (2015) |
| 2-Heptanol | green | –0.06 | 1321.0 | March *et al.* (2015) |
| 1-Hexanol | green | –4.49 | 1352.6 | March *et al.* (2015) |
| 1-Heptanol | green | –5.25 | 1369.9 | March *et al.* (2015) |
| (S)-2-Octanol | fatty | 0.43 | 1418.0 | March *et al.* (2015) |
| 1-Octen-3-ol | moldy | –6.5 | 1446.2 | March *et al.* (2015) |
| 2-Ethyl hexanol | citrus, fresh, floral, sweet | 1.17 | 1484.3 | Verzera *et al.* (2011) |
| 2-Nonanol | green | 0.39 | 1514.4 | March *et al.* (2015) |
| 2,3-Butanediol | fruity | 0.35 | 1531.2 | Sánchezpalomo *et al.* (2010) |
| Linalool | bergamot | –0.01 | 1540.6 | March *et al.* (2015) |
| 1-Octanol | soap | 1.69 | 1552.1 | March *et al.* (2015) |
| (S,S)-2,3-Butanediol | fruity | –2.54 | 1566.8 | Sánchezpalomo *et al.* (2010) |
| Terpinen-4-ol | woody, earthy | –1.96 | 1594.1 | Tietel *et al.* (2011) |
| 2-Furanmethanol | sugar burnt | 0.04 | 1647.2 | Feng *et al.* (2015) |
| 1-Nonanol | green | 0.04 | 1653.8 | March *et al.* (2015) |
| 3-Methylthio propanol | cooked potato | –0.65 | 1704.6 | Feng *et al.* (2014) |
| 1-Decanol | polished | –0.49 | 1756.1 | March *et al.* (2015) |
| Benzyl alcohol | floral, sweet | –0.26 | 1863.3 | |
| Phenylethyl alcohol | floral, rose-like | –12.01 | 1898.9 | Xiao *et al.* (2017) |
| 1-Dodecanol | raw carrot | 0.5 | 1963.1 | March *et al.* (2015) |
| 1-Undecanol | | 1.34 | 1963.5 | |
| Propanal | pungent | 0.04 | 793.86 | March *et al.* (2015) |
| 3-Methyl butanal | empyreumatic | –0.47 | 911.05 | Théron *et al.* (2010) |
| Pentanal | woody, fruity | 0 | 911.25 | Reboredo-Rodríguez *et al.* (2013) |
| Hexanal | cut grass | 1.43 | 1083.5 | Liu *et al.* (2015) |
| Heptanal | fatty | 4.86 | 1181.7 | March *et al.* (2015) |
| Octanal | watermelon | 2.5 | 1283.4 | March *et al.* (2015) |
| Nonanal | green, fresh | 2.32 | 1385.6 | Liu *et al.* (2015) |
| Decanal | waxy | 1.07 | 1488.9 | March *et al.* (2015) |
| Benzaldehyde | almond | 1.26 | 1506.6 | March *et al.* (2015) |
| Benzeneacetaldehyde | honey-like | –0.28 | 1626.4 | Feng *et al.* (2015) |
| 2,3-Butanedione | butter-lactic | 3.11 | 967.85 | Théron *et al.* (2010) |
| 2-Methyl-3-pentanone | | –0.09 | 990.73 | |
| 2,3-Heptanedione | | 0 | 1151.0 | |
| 2-Heptanone | banana | 0.99 | 1181.1 | March *et al.* (2015) |
| 3-Octanone | mushroom | –1.39 | 1251.3 | March *et al.* (2015) |
| Acetoin | buttery | –16.59 | 1279.2 | Carroll *et al.* (2016) |
| 2-Octanone | mushroom | –1.42 | 1280.8 | March *et al.* (2015) |
| 2-Nonanone | sweet | –1.66 | 1383.3 | Liu *et al.* (2015) |
| 2-Undecanone | floral | –3.13 | 1590.5 | March *et al.* (2015) |
| 2-Tridecanone | oily, nutty | –4.15 | 1801.9 | Reboredo-Rodríguez *et al.* (2013) |
| 2-Pentadecanone | | –3.19 | 2017.2 | |
| 2-Decanone | fruity | 0.63 | 2230.5 | March *et al.* (2015) |
| Acetic acid | sour | –7.73 | 1444.8 | Xiao *et al.* (2017) |
| Isobutyric acid | cheese | –3.09 | 1573.4 | March *et al.* (2015) |
| Isovaleric acid | cheese | –2.83 | 1675.7 | March *et al.* (2015) |

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Table 1. To be continued

| Compounds | Odour | Concentration changes | R.I. | References |
|---|---|---|---|---|
| Hexanoic acid | fatty, gammy, cheesy and dairy odours | –65.08 | 1838.2 | Resconi *et al.* (2017) |
| Heptanoic acid | cheesy | –1.89 | 1976.1 | March *et al.* (2015) |
| Octanoic acid | sweat, cheese | –98.01 | 2056.5 | Zhu *et al.* (2017) |
| n-Decanoic acid | waxy, fatty | –94.66 | 2276.5 | Resconi *et al.* (2017) |
| Dodecanoic acid | rubbery, musty | –10.8 | 2505.9 | Mahmoud & Buettner (2017) |
| Tetradecanoic acid | | 2.25 | 2711.6 | |
| Hexadecanoic acid | | –0.64 | 2902.3 | |
| Ethyl acetate | pineapple | 41.03 | 881.58 | Rayne & Forest (2016) |
| Ethyl propionate | fruit | 0.35 | 948.32 | Rayne & Forest (2016) |
| Ethyl isobutyrate | sweet, rubber | 0.19 | 957.99 | Rayne & Forest (2016) |
| Isobutyl acetate | banana | –0.35 | 1011.7 | Styger *et al.* (2011) |
| Ethyl butyrate | strawberry | 13.08 | 1037.1 | March *et al.* (2015) |
| Ethyl isovalerate | fruity | –0.04 | 1072.1 | Xiao *et al.* (2017) |
| Isoamyl acetate | banana | 3.46 | 1124.4 | Rayne & Forest (2016) |
| Ethyl valerate | yeast, fruit | 1.25 | 1136.3 | Rayne & Forest (2016) |
| Methyl hexanoate | fruit, fresh, sweet | –0.56 | 1185.7 | Rayne & Forest (2016) |
| Ethyl caproate | ripe fruits | 154.03 | 1232.9 | March *et al.* (2015) |
| Isoamyl butyrate | banana | 3.03 | 1263.5 | Pontes *et al.* (2012) |
| Hexyl acetate | banana | –0.46 | 1270.2 | March *et al.* (2015) |
| Propyl hexanoate | fruity | 0.74 | 1313.3 | Rayne & Forest (2016) |
| Ethyl heptanoate | overripe | 7.96 | 1330.1 | March *et al.* (2015) |
| Isobutyl hexanoate | fresh | 0.83 | 1349.6 | Rayne & Forest (2016) |
| Methyl octanoate | orange | –1.66 | 1384.7 | Rayne & Forest (2016) |
| Ethyl caprylate | sweet, fruity | 272.9 | 1430.9 | Sánchezpalomo *et al.* (2010) |
| Isopentyl hexanoate | apple, pineapple | 3.79 | 1453.8 | Zheng *et al.* (2014) |
| Ethyl nonanoate | waxy | –2.43 | 1529.6 | March *et al.* (2015) |
| Methyl caprate | | –1.97 | 1588.0 | |
| Ethyl caprate | sweet/fruity | 162.51 | 1632.9 | Sánchezpalomo *et al.* (2010) |
| 3-Methylbutyl octanoate | fruity | 0.04 | 1653.0 | Tabilio *et al.* (2013) |
| Ethyl benzoate | camomile, flower, celery, fruit | –6.54 | 1654.6 | Rayne & Forest (2016) |
| Ethyl decanoate | fruity | 14.87 | 1683.5 | Tabilio *et al.* (2013) |
| Ethyl undecanoate | cognac, coconut | 1.64 | 1734.6 | Rayne & Forest (2016) |
| Ethyl phenylacetate | sweet fragrance of honey | –0.01 | 1774.0 | Zhang *et al.* (2010) |
| Phenethyl acetate | sweet, honey-like | –2.27 | 1803.6 | Liu *et al.* (2015); Rayne & Forest (2016) |
| Ethyl myristate | sweet fruity, butter and fatty | 12.46 | 2048.6 | Tao *et al.* (2010) |
| Ethyl cinnamate | honey, cinnamon | –0.12 | 2122.2 | Rayne & Forest (2016) |
| Ethyl pentadecanoate | fruity | 0.75 | 2151.3 | Pino (2014) |
| Ethyl palmitate | incense wax smell, butter aroma | 13.66 | 2255.5 | Zhang *et al.* (2010) |
| Ethyl (*Z*)-9-hexadecenoate | waxy, buttery | 2.57 | 2281.3 | Pino (2012) |
| Ethyl laurate | leaf | 50.68 | 2458.7 | Rayne & Forest (2016) |
| Ethyl octadecanoate | waxy | 0.83 | 2458.9 | Pino (2012) |
| Ethyl oleate | fruity | –2.85 | 2477.3 | Moy *et al.* (2012) |
| Ethyl (9E)-9-octadecenoate | waxy | 15.49 | 2477.5 | Moy *et al.* (2012) |
| Ethyl linoleate | fruity | 7.58 | 2522.5 | Rayne & Forest (2016) and Moy *et al.* (2012) |
| 2-Pentylfuran | bean aroma, fruity | –0.94 | 1227.0 | Moy *et al.* (2012) |
| γ-Terpinene | citrus-like | 0.19 | 1238.0 | Kuraya *et al.* (2017) |
| 2,3,5-Trimethylpyrazine | burnt | –0.04 | 1398.5 | Feng *et al.* (2015) |
| 2-Acetylpyrrole | tea | –0.5 | 1957.5 | Joshi & Gulati (2015) |

Concentration changes calculated as: MJWB (40 h) – MJWB (0 h)

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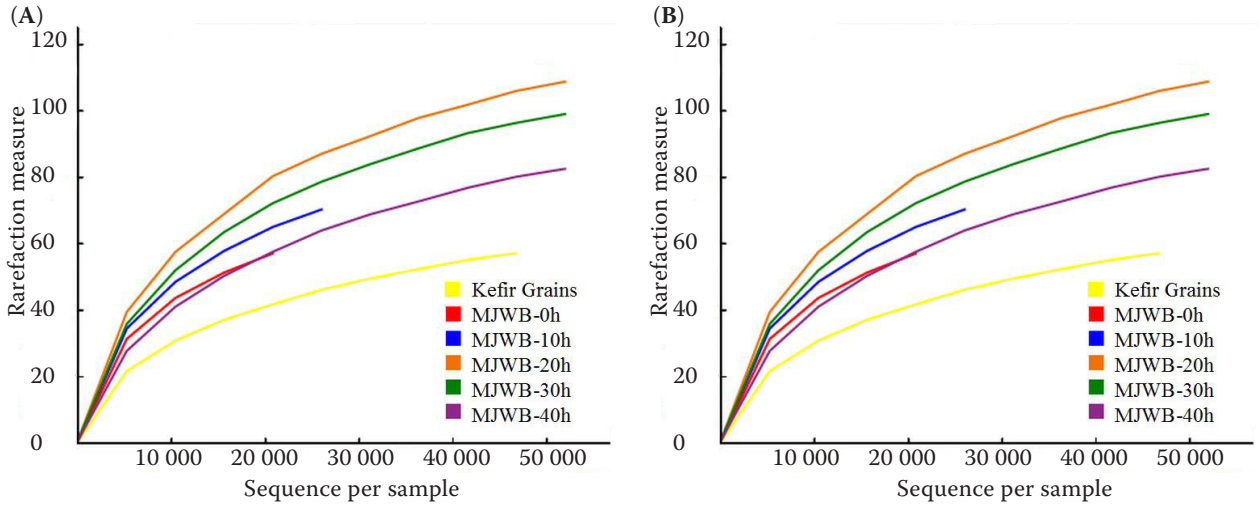


Figure 1. Rarefaction curves based on Illumina MiSeq sequencing of microbial communities in the fermentation process of mulberry juice-whey beverage: (**A**) bacteria and (**B**) fungi

***Overview of microbial community diversity.*** Using Illumina MiSeq sequencing, a set of more than 25 000 effective sequence tags was yielded for each sample (Table 2). Tags with 97% similarity were then grouped into OTUs by the CD-HIT clustering method. The rarefaction curves of bacteria and fungi did not approach saturation (Figure 1). The total number of bacterial OTUs (Table 2) estimated by Chao 1 estimator indicated that MJWB-20h had the greatest richness (showed the highest bacterial diversity). Similarly, kefir grains had the greatest richness for fungus (showed the highest fungal diversity). The Shannon diversity index provided how the abundance of each species was distributed in a community; the larger the Shannon index value, the higher the Alpha-diversity was acquired (Castelino *et al.* 2017).

***Microbial community structures of the groups.*** The RDP classifier was used to assign the sequence tags to different taxonomic levels (from phylum to genus) at 50% threshold. As shown in Figure 2, *Firmicutes*

Table 2. OUT-based diversity indexes of fermentation process in mulberry juice-whey beverage

| Groups | High-quality reads | Coverage rate | No. OUTs | Chao1[a] | Shannon[b] |
|---|---|---|---|---|---|
| **Bacteria** | | | | | |
| Kefir grains | 47507 | 94.66 | 17 | 159.0 | 1.74 |
| MJWB-0h | 23679 | 85.44 | 32 | 198.0 | 2.54 |
| MJWB-10h | 27283 | 83.49 | 34 | 246.0 | 2.94 |
| MJWB-20h | 57195 | 86.06 | 45 | 312.0 | 3.31 |
| MJWB-30h | 63841 | 87.61 | 36 | 299.0 | 3.07 |
| MJWB-40h | 89674 | 90.56 | 28 | 210.0 | 2.44 |
| **Fungus** | | | | | |
| Kefir grains | 47814 | 99.57 | 94 | 85.0 | 1.20 |
| MJWB-0h | 55926 | 99.40 | 79 | 58.0 | 0.73 |
| MJWB-10h | 61965 | 99.53 | 118 | 81.0 | 1.03 |
| MJWB-20h | 45296 | 99.69 | 76 | 41.0 | 1.50 |
| MJWB-30h | 27602 | 99.37 | 97 | 75.0 | 1.91 |
| MJWB-40h | 48926 | 96.66 | 73 | 47.0 | 1.43 |

[a]chaol richness estimator – a higher number indicates higher richness; [b]shannon index (H) – a higher value represents more diversity

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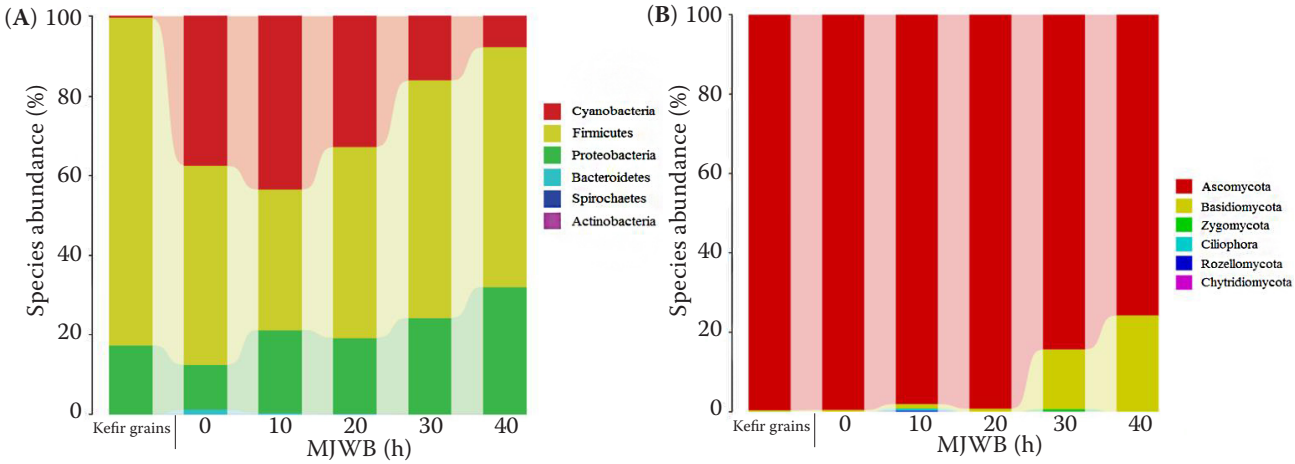


Figure 2. Bacterial (**A**) and fungal (**B**) community structure at the phylum level of fermentation process in mulberry juice-whey beverages

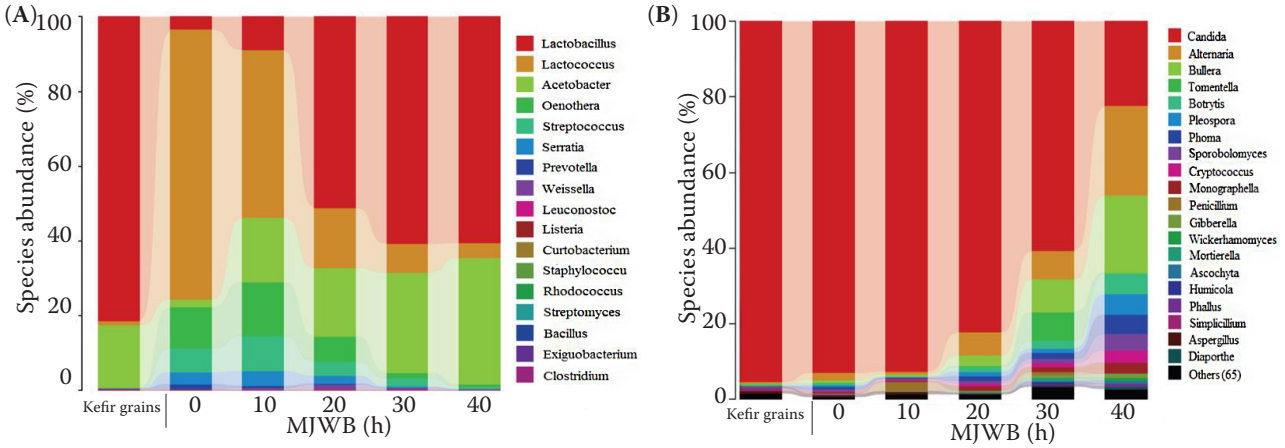


Figure 3. Bacterial (**A**) and fungal (**B**) community structure at the genus level of fermentation process in mulberry juice-whey beverages

(82.4%) were dominant bacteria in the Tibetan kefir grains. During the beverage fermentation process, *Firmicutes*, *Cyanobacteria* and *Proteobacteria* were dominant bacteria. Cyanobacteria increased from 37.5% at 0 h to 43.5% at 10 h and then decreased to 7.7% at 40 hours. Conversely, *Firmicutes* decreased from 50% at 0 h to 35.1% at 10 h and then gradually increased to 60.2% at 40 hours. Proteobacteria had a general trend of rise wave from 11.2% at 0 h to 32.1% at 40 hours. Other phyla were only < 1% during fermentation. For the fungal community structures, Ascomycota was the absolutely dominant fungus throughout fermentation while Basidiomycota was the second dominant fungal group at 0 and 10 hours.

At the bacterial family level, the majority of sequences belonged to 17 families (> 1% on average, Figure 3). *Lactobacillus* showed the highest abundances in kefir grains and *Acetobacter* was the second. *Lactococcus* was frequently detected due to the bad attachment of lactococcus to the kefir grains and entered into the beverage. This coincides with the results that Guzel-Seydim *et al.* (2010) obtained from a Turkey kefir using SEM (Guzel-Seydim *et al.* 2010). After 10 h fermentation, *Lactobacillus* rapidly proliferated after acclimatizing to the fermentation environment and *Lactococcus* rapidly decreased. *Acetobacter* was steadily increasing during the fermentation process. The ethanol produced by yeasts serves as a substrate for their growth and metabolism to acetic acid and, likewise, in vinegar production and production of beers of the lambic type. For the fungi, *Candida* was frequently detected with the highest abundances in almost all samples. *Candida*, *Alternaria* and *Bullera* had similar abundances at 0 h and then *Alternaria*

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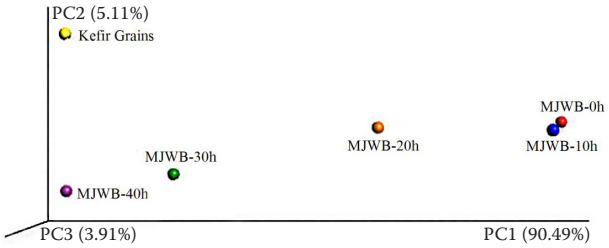


Figure 4. PCoA plot of the samples using unweighted UniFrac distance metric

and *Bullera* decreased with fermentation proceeded. Other fungi showed a similar tendency to *Alternaria* and *Bullera*.

The β-diversity analysis assesses differences between the microbial communities and reflects dissimilarity between the samples. A PCoA plot was used to visualize the data based on β-diversity metrics of unweighted UniFrac (Figure 4). The MJWB – 0 h and MJWB – 10 h were separated from the kefir grains. The samples along with the fermentation were close to kefir grains in the PcoA coordinate diagram, indicating that they had similar microbial communities. When the kefir grains were removed from whole milk, they should be washed with distilled water and then inoculated into the prepared mulberry juice-whey mixture. Some microbial cells, such as *Lactococcus,* were washed to the water, therefore, MJWB – 0 h and MJWB – 10 h had quite different microbial communities.

## CONCLUSIONS

Fermentation of mulberry juice and whey mixture by Tibetan kefir grains promoted considerable changes in the volatile flavour compounds and microbial communities as a result of bacterial and yeast growths during the fermentation process. Most of these compounds are similar to those reported for other juice whey fermented beverages. Higher alcohols and ethyl esters were the most dominant presented compounds. Illumina MiSeq sequencing showed that the communities changed during the fermentation process. *Lactobacillus* and *Candida* were frequently detected with the highest abundances during the later period of fermentation. Overall, our findings demonstrate the potential of using mulberry juice and whey to get the fermented kefir beverage. Further study should cover the evaluation of nutritional as well as organoleptic characteristics of the mulberry juice-whey beverages during refrigerated storage in order to assure that the product has acceptable quality for consumers.

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