Characterization of Microbial Community in Daqu by PLFA Method

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Daucu, as an essential saccharifying and fermentative agent during Chinese liquor manufacture, is closely related to the quality and yield of liquor. In order to explore the microbial community structure during the process of Daqu production, the phospholipid fatty acid (PLFA) patterns were analyzed. The results obtained by measuring the PLFA contents of pure mycelium and raw materials demonstrated that PLFA analysis was appropriate to explore the characteristics and shifts of the microbial community in Daqu sample. Analysis of the microbial community of Daqu by PLFA method suggested that fungi predominated the total amounts of biomass in Daqu. In addition, changes in contents of fungi and bacteria were also investigated during Daqu-making. From the shaped stage to the finished stage of Daqu, fungi dominated gradually, and a small amount of bacterial biomass were detected in all finished Daqu samples. In three finished Daqu samples, the fungal biomass accounted for 68.85% – 80.11% of total biomass. Furthermore, the contents of gram-positive bacteria were higher than that of gram-negative bacteria in all Daqu samples.

Keywords: Daqu, phospholipid fatty acids, microbial community, fungal biomass

Introduction

Chinese liquor is one of the well-known distilled spirits in the world, and the manufacture technique is unique. Daqu is an essential starter culture during the production of Chinese liquor and has noteworthy impact on the flavor of the product (Xu et al., 2010). Based on the aromas of distillate, several types of Daqu can be distinguished, such as light-flavor, strong-flavor and soy sauce-flavor, which depend on different manufacture techniques. Daqu is a complex substrate throughout the fermentation of grain via a natural inoculation of microbial communities originated from production environment. Therefore, microbial community and the metabolites are closely related to incubation conditions and environments (Zhang et al., 2009). Although the production methods of various types of Daqu are slightly different, the process often involves three stages: material shaping, incubating (particularly, microbial community forming) and drying (decreased to 13 – 14% of humidity by natural drying). The particular microbial community forming stage is the most important process for the production of various necessary enzymes and flavor compounds. For this reason, it is necessary to investigate the characteristic of microbial flora as well as their shifts to understand the relationship between microbial community and metabolites, and optimize the culture conditions (Wu et al., 2009a). Recently, some cultivable studies on microbial community during the process of Daqu manufacture have been reported (Wang et al., 2008). Whereas, it was not only time-consuming, but also difficult to obtain the microbial community structure effectively, since 80 – 99% of microorganisms were uncultured (Amann et al., 1995). PCR-based fingerprinting technique, providing a higher resolution and more information about the changes in the whole microbial community structure, has been applied to investigate the Daqu microbial community (Wang et al., 2011; Xiu et al., 2012),

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and various unreported microorganisms have been detected. Zheng et al. (Zheng et al., 2012a) investigated the microbiota of Fen-Daqu by culture-dependent and culture-independent methods, and the results showed that a total of 190 microbial strains comprising 109 bacteria and 81 yeasts and moulds were isolated and identified. In addition, both approaches revealed that *Bacillus licheniformis* was an abundant species, and *Saccharomyces fibuligera, Wickerhamomyces anomalus,* and *Pichia kudriavzevii* were the most common yeasts encountered in Fen-Daqu. Xiu et al. (Xiu et al., 2012) demonstrated the microbial diversity of *Maotai-Daqu* by using nested PCR-DGGE, and the microbial distributions during the periods of shaping, ripening and drying in Daqu-making. These results may contribute to the design of specific starter or adjunct cultures. However, some unquantifiable biases were noticed (e.g. originated from DNA extraction, uneven lysis or PCR amplification) (Blackwood et al., 2007). In this manuscript, the phospholipid fatty acids (PLFAs)-based method was employed to assess the microbial community in Daqu.

As a key membrane component in all living cells, phospholipid was not found in dead cell or storage compounds (Zelles, 1997), and phospholipid fatty acids were widely accepted as biomarkers to indicate viable microbial biomass and provide a microbial community ‘fingerprint’ (Liang et al., 2008). Recently, PLFA analysis is becoming increasingly used in microbial ecology studies in soil, compost and sediment (Farrell et al., 2010; Klamer and Bäath, 2004; Müller et al., 2010). However, to our knowledge, no report was available about the application of PLFA method in Daqu analysis. Therefore, the purpose of this study was to assess the feasibility of PLFA analysis and investigate the microbial community of Daqu based on PLFA method. Results presented in this manuscript may help to obtain a better understanding of the microflora in Daqu and subsequently improve the quality of Chinese liquor.

Materials and Methods

**Daqu samples** Daqu samples were collected from three famous liquor-production factories in Sichuan province, China (Daqu LJ, collected from Luzhou Laojiao Co., Ltd; Daqu FG, collected from Fenggu distillery Co., Ltd; Daqu XF, collected from Xufu distillery Co., Ltd). The samples were harvested at different periods during the culture process, and at least 3 Daqu samples were collected at each period (Fig. 1). The monoblock brick-shaped Daqu samples were smashed and mixed uniformly. About 150 g of each composite sample was stored in polythene bags at -20°C (no more than 24 h) for further analysis.

**Strains and growth conditions** Fungi used in this experiment were selected based on the fungal characteristic in Daqu (Zheng et al., 2012b). *Mucor* MZ1-24 and *Absidia corymbifera* CQB43 were isolated from bran vinegar Daqu, which were identified as Mucor and *Absidia corymbifera,* respectively, based on morphological analysis and biochemical characteristics. *Aspergillus niger* CGMCC3.324 and *Aspergillus oryzae* CGMCC3811 were purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China), while *Rhizopus nigricans* SICC3.26 and *Rhizopus* sp. SICC3.107 were purchased from Sichuan Microbiological Resources Infrastructure & Culture Collection Center (SICC, Chengdu, China). In order to avoid the interference of the substrate on the PLFAs profile of microbial cell membrane, all strains used were grown on grain agar (20 g Daqu raw material (wheat: pea = 8:1); 200 g water; 4.4 g agar; 121°C autoclaving for 20 min) covered with cellophane at 30°C for 72 h. Mycelia of the inoculated strains were collected from the surface of the cellophane covered on the grain substrate and the PLFAs contents were analyzed.

**Extraction and analysis of PLFA** Lipids were extracted with a single-phase chloroform-methanol-phosphate buffer described by Wu et al (Wu et al., 2009b). Samples (Daqu sample 1 g, mycelium sample 0.1 g) were extracted with a single-phase mixture of chloroform: methanol: phosphate buffer (15.2 mL at 1:2:0.8 volume basis) in a horizontal shaker (250 × g) at room temperature for 2 h. The single-phase system was split into two phases by adding phosphate buffer (4.8 mL) and chloroform (6 mL) and left overnight to separate. The CHCl₃ layer was then transferred to a new tube and dried under N₂ at 30°C. According to the high content of free fatty acid in Daqu sample, a NH₂-Silica solid phase extraction column was used in this experiment. The total lipids contained in the chloroform phase were fractionated into neutral lipids, free fatty acid, and phospholipids on a solid phase extraction column (NH₂-Silica, 200 mg, 3 mL, Welchrom) by eluting them sequentially with 10 mL isopropanol-chloroform solution (1:2), 10 mL 2% acetic acid in ether, and 10 mL methanol, respectively.

The phospholipid fraction was methylated to fatty acid methyl ester by a mild alkaline methanolsysis (Dowling et al., 1986). In
short, 1 mL methanol-toluene solution (1:1) and 1 mL 0.2 M KOH-methanol were added, and the mixture were heated at 37°C for 30 min. After methylation, distilled water (2 mL) and acetic acid solution (1 M, 0.3 mL) were added. Fatty acid methyl esters were extracted in hexane (2 mL) twice and dried under N₂. Fatty acid methyl esters (FAMEs) were analyzed by a gas chromatogram/mass spectrometry system (Thermo Fisher Corporation, USA). The GC system was equipped with a TR-5MS (30.0 m × 320 µm × 0.25 µm) capillary column. Identifications of FAMEs were compared with the standards (C4-24, Accustandard, USA) and mass spectrum (NIST05). The methyl nonadecanoate fatty acid (19:0, Accustandard, USA) was added as an internal standard. To remove lipid contaminants, all glassware used was heated overnight at 500°C. Standard nomenclature is used to describe FAMEs (Federle et al., 2006; Frostegård and Bååth, 1996; Moore-Kucera and Dick, 2008; Olsson and Alström, 2000) and the PLFAs characteristic of Gram-positive bacteria (GM+), Gram-negative bacteria (GM−), and fungi were listed in Table 1. All values of PLFA concentrations were the mean of at least three independent extraction processes.

Statistical analysis Molar amounts [nmol/g (dry weight, dw)] of the individual PLFAs were used as an estimation of microbial biomass. The relative proportions of PLFAs expressed as arithmetic means ± standard deviations (n = 3) were used to describe the microbial community structure. Analysis of variance (One-way ANOVA) with Duncan’s test (P < 0.05) was used to represent the significant differences of each PLFA data by SPSS 17.0 software (SPSS Inc. IL, USA).

Results

PLFAs content of mycelium Table 2 displayed the phospholipid fatty acids content of various fungal strains. As shown in Table 2, the profiles of PLFAs and their contents were significantly different (14.43 – 42.29 µmol/g dw) in the mycelium of various strains tested. The predominant PLFAs were 16:0, 18:2ω6,9 and 18:1ω9, and the latter two were often used as the indicator of fungal biomass (Klamer and Bååth, 2006). The ratios of the specific PLFAs to total PLFAs were different in all strains. As for Aspergillus (CGMCC3.324 and CGMCC3811), the ratios of 18:2ω6,9 were 39.2 mol% and 53.1 mol%, while the ratios of 18:1ω9 were 26.9 mol% and 12.8 mol%, respectively. As for Rhizopus (SICC3.107 and SICC3.26), the ratios for 18:2ω6,9 were 37.5 mol% and 37.3 mol%, respectively. In addition, 18:3ω6,9,12 was only measured in Mucoraceae, and PLFA 18:2ω9,12 was only detected in Mucor. A detailed analysis of the results presented in Table 2 showed that similar proportions of fungi biomarkers (18:1ω9, 18:2ω6,9 and 18:3ω6,9,12) were observed for these strains. These results suggest that the analysis of specific biomarker (18:1ω9, 18:2ω6,9 and 18:3ω6,9,12) may be a feasible method to evaluate the biomass of fungal strains.

Comparison of PLFAs in the substrates before and after cultivation As shown in Table 3, eight PLFAs were detected in the substrate before cultivation, which consisted of five saturated, two monounsaturated and one polyunsaturated fatty acid, while only four PLFAs were detected in the substrate after cultivation. After cultivation, primary PLFAs were unsaturated such as 18:2ω6,9 and 18:1ω9, and the saturated PLFAs 14:0, 15:0 and 17:0 were reduced below the detection limit. In addition, the contents of 16:0 and 18:0 in cultured substrate were reduced by 40.5% and 50.0%, respectively. Although the content of total PLFAs was decreased, no obvious change was found in the proportions of PLFA 18:2ω6,9 and 18:1ω9 (63.0% and 67.2% for the proportions before and after cultivation, respectively).

Table 2 showed that similar proportions of fungi biomakers (18:1ω9, 18:2ω6,9 and 18:3ω6,9,12) were observed for these strains. These results suggest that the analysis of specific biomarker (18:1ω9, 18:2ω6,9 and 18:3ω6,9,12) may be a feasible method to evaluate the biomass of fungal strains.

PLFA profiles of Daqu samples Figure 2 showed the PLFA profiles of three different Daqu samples, and these samples exhibited similar profiles of PLFA. The dominant PLFAs of these Daqu samples were 16:0, 18:2ω6,9 and 18:1ω9, and these PLFAs amounted for more than 95% of total PLFAs during the shaped period of Daqu (Fig. 2). With the extension of incubation time, differences in PLFAs species and total amounts were observed. 9, 11, 15 PLFAs were detected during the ripening period in LJ, FG, and XF Daqu samples, respectively, and the total amounts of PLFA increased 2.40-, 2.51-, and 2.16-fold compared with that determined during the shaped period. Especially, the contents of 18:2ω6,9 and 18:1ω9 enhanced 2.95-, 2.96-, and 2.73-fold in LJ, FG, and XF Daqu, respectively. For finished Daqu, 10, 11 and 15 PLFAs were detected in LJ, FG, and XF Daqu, and the total PLFA amounts were 1645.21, 1904.91, and 1503.53 nmol/g (dw), respectively.

Table 1. PLFA marker used for taxonomic microbial groups

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>PLFA group</th>
<th>Specific PLFA markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Multiple groups</td>
<td>a14:0, i16:0, 16:1ω9</td>
<td>(Frostegård and Bååth, 1996, Moore-Kucera and Dick, 2008)</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>Branched PLFAs</td>
<td>i14:0, i15:0, i16:0, i17:0, a15:0, a17:0</td>
<td>(Moore-Kucera and Dick, 2008, Olsson and Alström, 2000)</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>Cyclopropyl and monounsaturated PLFAs</td>
<td>16:1ω9, 16:1ω5, 16:1ω7, cy17:0</td>
<td>(Moore-Kucera and Dick, 2008, Olsson and Alström, 2000)</td>
</tr>
<tr>
<td>Fungi</td>
<td>Polyunsaturated PLFAs</td>
<td>18:1ω9, 18:2ω6,9, 18:3ω6,9,12</td>
<td>(Federle et al., 2006)</td>
</tr>
</tbody>
</table>
### Table 2. Phospholipid fatty acids content of various fungal strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>PLFA content$^1$ (µmol/g dw )</th>
<th>14:0</th>
<th>15:0</th>
<th>16:1ω7</th>
<th>16:0</th>
<th>17:0</th>
<th>18:3ω6,9,12</th>
<th>18:2ω9,12</th>
<th>18:2ω6,9</th>
<th>18:1ω9</th>
<th>18:0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td></td>
<td>ND$^2$</td>
<td>0.90 ± 0.19d</td>
<td>ND</td>
<td>12.15 ± 1.52b</td>
<td>0.2 ± 0.04</td>
<td>ND</td>
<td>ND</td>
<td>16.58 ± 4.24d</td>
<td>11.39 ± 1.96c</td>
<td>0.66 ± 0.23b</td>
<td>42.29 ± 3.95e</td>
</tr>
<tr>
<td>CGMCC3.324</td>
<td></td>
<td>ND</td>
<td>0.09 ± 0.03ab</td>
<td>ND</td>
<td>5.98 ± 1.79a</td>
<td>ND</td>
<td>2.07 ± 0.19b</td>
<td>ND</td>
<td>9.60 ± 0.67c</td>
<td>7.32 ± 1.35b</td>
<td>0.54 ± 0.10ab</td>
<td>25.61 ± 3.85d</td>
</tr>
<tr>
<td><em>Rhizopus sp.</em></td>
<td></td>
<td>ND</td>
<td>0.27 ± 0.05c</td>
<td>ND</td>
<td>6.45 ± 0.97a</td>
<td>ND</td>
<td>1.16 ± 0.16a</td>
<td>ND</td>
<td>3.73 ± 0.42ab</td>
<td>7.71 ± 0.82b</td>
<td>ND</td>
<td>19.05 ± 0.27bc</td>
</tr>
<tr>
<td>SICC3.107</td>
<td></td>
<td>ND</td>
<td>0.17 ± 0.03bc</td>
<td>0.13±0.02</td>
<td>5.21 ± 0.61a</td>
<td>ND</td>
<td>1.29 ± 0.26ab</td>
<td>ND</td>
<td>6.51 ± 0.81bc</td>
<td>3.52 ± 0.38a</td>
<td>0.58 ± 0.13ab</td>
<td>17.47 ± 1.40ab</td>
</tr>
<tr>
<td><em>Absidia corymbifera</em></td>
<td></td>
<td>ND</td>
<td>0.80 ± 0.19b</td>
<td>ND</td>
<td>4.35 ± 0.74a</td>
<td>ND</td>
<td>4.39 ± 0.49e</td>
<td>1.01 ± 0.12</td>
<td>1.36 ± 0.01a</td>
<td>10.00 ± 0.09c</td>
<td>0.35 ± 0.04a</td>
<td>22.28 ± 0.88cd</td>
</tr>
<tr>
<td>CGMCC3811</td>
<td></td>
<td>ND</td>
<td>0.15 ± 0.004</td>
<td>0.20 ± 0.009</td>
<td>0.12 ± 0.004</td>
<td>35.43 ± 3.04</td>
<td>0.15 ± 0.005</td>
<td>51.62 ± 4.03</td>
<td>11.37 ± 0.15</td>
<td>0.96 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizopus nigricans</em></td>
<td></td>
<td>ND</td>
<td>0.15 ± 0.004</td>
<td>0.20 ± 0.009</td>
<td>0.12 ± 0.004</td>
<td>35.43 ± 3.04</td>
<td>0.15 ± 0.005</td>
<td>51.62 ± 4.03</td>
<td>11.37 ± 0.15</td>
<td>0.96 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SICC3.26</td>
<td></td>
<td>ND</td>
<td>32.10 ± 3.03</td>
<td>ND</td>
<td>56.81 ± 4.16</td>
<td>10.36 ± 0.66</td>
<td>0.73 ± 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Mucor</em></td>
<td></td>
<td>ND</td>
<td>32.10 ± 3.03</td>
<td>ND</td>
<td>56.81 ± 4.16</td>
<td>10.36 ± 0.66</td>
<td>0.73 ± 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MZ1-24</td>
<td></td>
<td>ND</td>
<td>32.10 ± 3.03</td>
<td>ND</td>
<td>56.81 ± 4.16</td>
<td>10.36 ± 0.66</td>
<td>0.73 ± 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different letters represented significant differences (ANOVA with Duncan’s test, $P < 0.05$)

$^1$Mean ± SD, (n = 3)

$^2$ND, not determined

### Table 3. Comparison of fatty acids proportion in substrate before and after incubation.

<table>
<thead>
<tr>
<th>Fatty acid component (%)</th>
<th>14:0</th>
<th>15:0</th>
<th>16:1ω7</th>
<th>16:0</th>
<th>17:0</th>
<th>18:2ω6,9</th>
<th>18:1ω9</th>
<th>18:0</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A</em> substrates before incubation</td>
<td>0.15 ± 0.004</td>
<td>0.20 ± 0.009</td>
<td>0.12 ± 0.004</td>
<td>35.43 ± 3.04</td>
<td>0.15 ± 0.005</td>
<td>51.62 ± 4.03</td>
<td>11.37 ± 0.15</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td><em>B</em> substrates after incubation</td>
<td>0.15 ± 0.004</td>
<td>0.20 ± 0.009</td>
<td>0.12 ± 0.004</td>
<td>35.43 ± 3.04</td>
<td>0.15 ± 0.005</td>
<td>51.62 ± 4.03</td>
<td>11.37 ± 0.15</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td><em>C</em> ND, not determined</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ substrates before incubation

$^b$ substrates after incubation

$^c$ ND, not determined
Microbial community structure of Daqu samples  Changes in microbial community structure of Daqu sample during the process of Daqu manufacture were investigated (Fig. 3). No significant difference was observed in total biomass of all samples in the initial state of Daqu-making. The biomass increased from shaped period to ripened period and then slightly decreased in finished phase. In the initial stage of Daqu production process, the PLFAs resulted from wheat cell and some endophytic fungus. With the extension of cultivation time, microorganisms originated from the environment propagated gradually and formed particular microbial community. During the ripened stage of cultivation, the fungi dominated in all of the Daqu samples, and the proportion of the bacteria PLFAs was slightly increased. As for finished Daqu, the fungi were still dominant in three kinds of Daqu, which accounted for 74.46%, 80.11% and 68.85% of total PLFAs amounts in LG, FG, and XF samples, respectively. In addition, in all samples, the contents of gram-positive bacteria were higher than that of gram-negative bacteria, which accounted for 4.72%, 3.80%, and 5.83% of total biomass in LG, FG, and XF Daqu samples, respectively.

Discussion

In the present study, microbial community structures in Daqu sample were determined by using PLFA method. Phospholipid fatty acid, the biomarker of living microorganisms, is not found in dead cell or storage compounds (Zelles, 1997). Certain PLFA biomarkers are related to specific groups of microorganism. Thus, PLFA method have effectively applied to characterize the shifts of the microbial community structure (Bääth et al., 2005). However, some studies reported that the phospholipids component of cell membrane were rapidly metabolized following cell death, but this conclusion obtained by particular experimental conditions (Hill et al., 2000). Furthermore, the PLFA biomarkers 18:1ω9, 18:2ω6,9 and 18:3ω3,6,9 commonly used as fungal biomarkers in soil have also been found in plant tissues (the raw materials of Daqu-making) (Laczko et al., 2003). Therefore, it is necessary to investigate the feasibility of PLFA method on determining the Daqu microbial community structure, as wheat and pea are main materials during Daqu manufacture.

6 fungal strains, which were common microorganisms during Daqu production, were selected for PLFA analysis. The specific PLFAs of fungi (18:1ω9 and 18:2ω6,9, and 18:3ω3,6,9) accounted for more than 90% of total PLFA excluding 16:0. The results suggested that specific biomarkers may be a possible method to determine the biomass of fungal strains. In addition, the fungal biomarkers PLFA 18:1ω9 and 18:2ω6,9 were detected in the substrate before and after cultivation, and the result indicated that the fungal biomass and percentage (mol%) may be overestimated by the sum of PLFA 18:1ω9, 18:2ω6,9 and 18:3ω3,6,9. Pre-treatments such as sieving and/or hand-picking were used to eliminate the biomarkers PLFA originated from plant cell contributed to soil samples (Kaiser et al., 2010). But it was almost impossible to eliminate the interference of plants PLFAs to fungal PLFAs biomarkers by this manner since wheat and bran were essential substrates for Daqu manufacture. Nonetheless, the PLFAs concentration in culture mediums during the incubation process (incubated at 30°C for 72 h) had significant decrease (more than

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**Fig. 2.** PLFAs composition of Daqu samples collected from different periods. (A) LJ Daqu; (B) FG Daqu; (C) XF Daqu. Error bars indicate standard deviations ($n = 3$).
37.5%, abiotic and/or biotic), which indicated that phospholipids were unstable in this process. The complete turn-over time for PLFAs in Daqu producing was not known, but previous study showed a short turn-over time for phospholipids in sediments (White et al., 1979). And this degradation was thought to be fairly rapid in natural environments (King et al., 1977; Klamer and Bååth, 2006). As for the long process (20 ~ 40 d) and the shift of temperature (30 ~ 65°C) during the Daqu production, the contribution of PLFAs from raw material to microorganism PLFAs biomarkers in Daqu could be neglected. There was other evidence from our previous studies, which showed that the high correlation ($R = 0.987$, $P < 0.05$) between ergosterol (another fungal biomarker) and PLFA (18:2o6,9) content in Daqu samples was obtained (Qin et al., 2011). In addition, previous PLFAs analysis concerning decomposition of rice straw (Nakamura et al., 2003) and the microbial communities of fermented grains (Zheng et al., 2011), had shown that none/low concentration of PLFAs 18:2o6,9 and 18:1o9 was detected, although the great quantity of plant cell existed. According to these results, it was suggested that high concentration of PLFAs 18:2o6,9 and 18:1o9 in Daqu samples were accounted for fungal mycelium, and PLFA analysis could give more information of living microbes compared with other methods.

Changes in microbial community structure in the process of Daqu production were also investigated. The fungi of Daqu represent higher amounts of biomass than the bacteria. It should be realised however that since fungi biomass is larger than that of bacteria, this does not imply that fungi have a higher number or larger metabolic impact on Daqu (Zheng et al., 2012b). During the shaped phase of Daqu, the PLFA biomarkers of both GM+ and GM- were not detected, and the fungal PLFAs may originate from the raw materials of Daqu. With the increase of incubation time, the biomass of fungi increased sharply, while the contents of bacteria increased gradually, and fungi still dominated the microflora. During the process of Daqu-making by solid-state fermentation, the temperature rose sharply due to the limitation of mass and heat transfer, and only the thermophile strains survived. Xiu et al (Xiu et al., 2012) determined the microbial diversity in Maotai-Daqu, and the results showed that Bacillus and lactic acid bacteria were the dominant bacteria during the ripening period of Daqu-making. Bacillus can survive in a low moisture content (13 ~ 14%) and high temperature (60 ~ 62°C) (Shi et al., 2009). Lactic acid bacteria usually act as a regulator of microbial consortia diversity in a variety of food and may contribute to the formation of flavour compounds by producing a variety of enzymes (Liu et al., 2011). During the finished period of Daqu-making, the percentage of PLFA biomarkers of bacteria in the finished Daqu was still very low compared with that of fungi. When the amounts of bacterial PLFA were converted into the number of bacterial cells, the average amount of bacterial PLFA was $1.4 \times 10^{17}$ mol/ cell (Frostegård and Bååth, 1996). With this conversion factor, we could estimate that bacterial biomass in the finished Daqu samples were from $9.8 \times 10^9$ to $10.94 \times 10^9$ cells/g (dw). These results were a little higher than that detected from culture method ($6.3 \sim 21.8 \times 10^7$ cfu/g (dw)) (Wang et al., 2008). This may be ascribed to the fact that some uncultured bacteria existed which could not be responded by culture-dependent method (Wang et al., 2011). In addition, this high level of bacterial biomass in Daqu sample makes this starter different from other Asian traditional alcoholic fermentation starters such as Men and Ragi of which the bacterial loads of $2.6 \sim 6.2$ Log cfu/g and $4.3 \sim 5.8$ Log cfu/g, respectively, represented mainly lactic acid bacteria (Hesseltine et al., 1988; Thanh et al., 2008). Of

**Fig. 3.** Changes of microbial community structure during the process of Daqu production. Panels A, B, and C represent PLFAs concentration characterizing GM+, GM-, and fungi, respectively. LJ, FG, and XF present LJ Daqu, FG Daqu, and XF Daqu, respectively. Different letters indicate significant differences ($P < 0.05$, ANOVA, Duncan’s test). Error bars indicate standard deviations ($n = 3$).
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course, the different types of Daqu samples and the applicability of this conversion factor (calculated from soil bacteria) may also be explored further.

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