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ORIGINAL ARTICLE

# Effect of temperature on microbial composition of starter culture for Chinese light aroma style liquor fermentation

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**Significance and Impact of the Study**: The usage of a combination of three types of *Daqu* including Qingcha, Hongxin and Houhuo as starter culture is an important characteristic of production technology of Chinese light aroma style liquor. Micro-organisms from the environment naturally inoculated in *Daqu* are propagated to form the special microbial community under the control of several operating parameters, especially temperature, and finally play various roles in the fermentation process of liquor. An in-depth study of the relationship between incubation temperature and microbiota in *Daqu* during the manufacturing is fundamental to understand this complicated process and to prepare high-quality starter culture for fermentation.

**Keywords**
heat tolerant, light aroma style liquor, microbial composition, starter culture, temperature.

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## Abstract

Light aroma style liquor is one of the basic types of Chinese liquor and is produced with a special technique of using a combination of three types of *Daqu* as starter culture. The succession of incubation temperature, a main operating parameter, and microbial composition in *Daqu* were investigated during the manufacturing process. The most significant difference in temperature occurred during the middle stage at which the highest and the lowest temperatures were detected in Houhuo (HH) and Qingcha (QC), respectively. It was shown that for the counting data, the population of fungi was identical and that of bacteria was different between three types of *Daqu*. According to analysis results of microbial community structure using PCR-denaturing gradient gel electrophoresis (PCR-DGGE), lactic acid bacteria were one of the dominant bacterial groups in all of *Daqu* and fungal diversity in QC was higher than that in HH and Hongxin (HX). The difference in incubation temperature led to the accumulation of different heat-tolerant and heat-sensitive fungi in the completed *Daqu*. PCA of DGGE profiles revealed that microbial community structure was distinct between three types of *Daqu*. It was presumed that temperature might play a decisive role in the formation of micro-organism composition in starter cultures.

## Introduction

Liquor is a traditional distillate spirit, emblematic of Chinese culture. Naturally fermented *Daqu* is used as starter culture and provides micro-organisms, enzymes and compounds for the following liquor fermentation. The manufacturing process of *Daqu* is shown in Fig. 1.

Micro-organisms are vitally important for *Daqu* quality, which originate from the environment including raw materials, water, air and production utensils. Micro-organisms in *Daqu* comprise bacteria, yeast and moulds, playing diverse roles in the brewing of liquor. Bacteria are important sources of proteases and amylases and produce a large number of organic acids (Li *et al.* 2009; Lei 2011). Yeast synthesize a serial of metabolites such as esters, acids, alcohols and aldehydes (Wu *et al.* 2012). Moulds are the main saccharifying micro-organisms and also form some esters and other substances to improve liquor quantity (Yang *et al.* 2013). The microbiota of *Daqu* has been analysed using culture-dependent and culture-independent methods

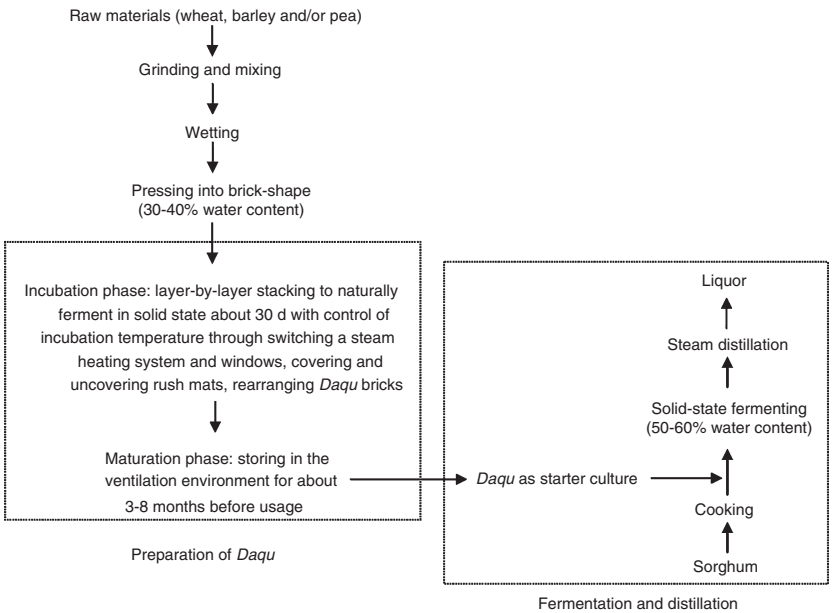


**Figure 1** The manufacturing process of *Daqu* and its role in the production of liquor.

(Hui *et al.* 2009; Luo *et al.* 2009; Zheng *et al.* 2011, 2013; Liu *et al.* 2012; Wu *et al.* 2013; Chen *et al.* 2014). The identified bacteria existing in *Daqu* include *Bacillus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Clostridium*, *Staphylococcus*, *Microbacterium*, *Acetobacter* and actinomycetes. The genera of yeast contain *Saccharomyces*, *Pichia*, *Candida*, *Zygosaccharomyces*, *Hanseniaspora*, *Debagryomyces*, *Endomycopsis*, *Saccharomycopsis*, *Issatchenkia*, *Rhodotorula*, *Trichosporon*, *Torulaspora* and *Schizosaccharomyces*. The main moulds are *Absidia*, *Aspergillus*, *Rhizopus*, *Mucor*, *Penicillium*, *Paecilomyces* and *Monascus*.

The comparison results of microbial community in *Daqu* which were produced in different regions using different production techniques revealed that several factors jointly influence micro-organism composition in *Daqu* and temperature plays a more important role than geographical conditions (Wang *et al.* 2011). According to the highest incubation temperature, *Daqu* is usually classified into three categories, low-temperature *Daqu* (40–50°C), medium-temperature *Daqu* (50–60°C) and high-temperature *Daqu* (60–65°C), and the different microbial compositions exist between each type of *Daqu*.(Zheng *et al.* 2011).

Light aroma style liquor is one of the basic liquor styles and its production technique has a special characteristic to utilize a combination of three types of *Daqu* as starter culture, which belong to low-temperature *Daqu* and are usually named as Qingcha (QC), Hongxin (HX) and Houhuo (HH). The manufacturing techniques of these three kinds of *Daqu* are very similar involving raw materials, pretreatment and incubation environment, except for the incubation temperature. During the manufacturing process, temperature alters micro-organism growth status and then affects microbial community structure and metabolite profile in *Daqu*, such as the earthy odour geosmin and its producing bacteria (Du and Xu 2012; Du *et al.* 2013). However, microbial composition in *Daqu* used in the production of light aroma style liquor has not been comprehensively analysed and was merely investigated in one type of *Daqu* (Zheng *et al.* 2012, 2013) or matured *Daqu* (Shi *et al.* 2009; Zhang *et al.* 2014).

The aim of the present research was to elucidate the effect of temperature on the formation of microbial community in *Daqu*. For this purpose, the succession of incubation temperature and microbial composition during the manufacturing process of three types of *Daqu* was monitored, respectively.

## Results and discussion

### Temperature dynamics during the manufacturing process of *Daqu*

Figure 2 presents the change in temperature during the making process of *Daqu*. The initial temperatures of three types of *Daqu* were close to the room temperature. In the first 4 days, the temperatures increased quickly from 20°C to nearly 40°C. Then, the rate of temperature increase slowed down, and at the 12th day, the incubation temperature reached the maximum value, which was 44, 46 and 48°C inside the piles of QC, HX and HH, respectively. In the later process, pile temperature gradually decreased till the end. There was a significant difference between incubation temperature in the manufacturing process of three types of *Daqu* ($P < 0{\cdot}05$), except the fourth day.

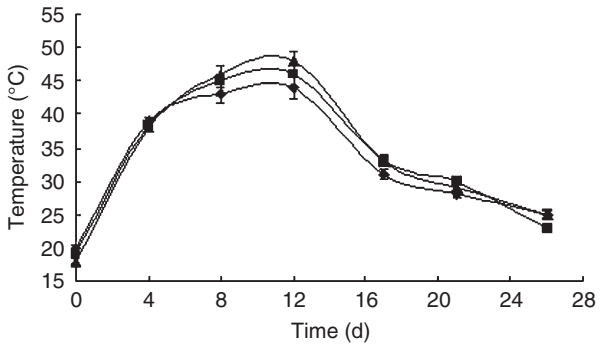


**Figure 2** Incubation temperature curve during the manufacturing process. GC (♦), HX (■) and HH(▲).

### Monitoring of microbial counts during the preparation of *Daqu*

*Daqu* was exposed to natural environment and micro-organisms mainly from air inoculated and propagated in *Daqu* to form the special microbial community. Contents of bacteria, yeast and moulds in *Daqu* were enumerated using the plate-counting method.

As shown in Fig. 3, there were about $9{\cdot}29 \pm 0{\cdot}45 \times 10^5$ CFU $g^{-1}$ of bacteria, $1{\cdot}69 \pm 0{\cdot}098 \times 10^3$ CFU $g^{-1}$ of moulds and dozens of yeast in *Daqu* at the beginning. Yeast and moulds in *Daqu* quickly propagated in the former 4 days. Hereafter, the number of yeast decreased continuously until incubation temperature fell near to 30°C (Fig. 3b) and mould content appeared a short-term decline (Fig. 3c). This may be because the suitable growth temperature of yeast is ordinarily between 20 and 30°C and moulds can form spores to resist higher temperatures. The similar tendency of fungi resulted from their temperature sensibility, and only mould content in the eighth-day *Daqu* showed significant difference ($P < 0{\cdot}05$). On the contrary, bacteria can grow in a wide-temperature range and its amount gradually increased in *Daqu* (Fig. 3a). The counting data exhibited a significant difference from the fourth to the 16th days ($P < 0{\cdot}05$). Traditional enumeration methods could not distinguish the difference in microbial quantity between three types of completed *Daqu,* and there were $10^9$ CFU $g^{-1}$ of bacteria and moulds and $10^6$ CFU $g^{-1}$ of yeast.

### Microbial community succession in *Daqu*

The microbial population successions during the manufacturing process were detected by isolating DNA from *Daqu* taken at different periods and then performing DGGE analysis using 16S rRNA, 18S rRNA and 26S rRNA as target genes, respectively. Distinct DNA bands were extracted from DGGE gels, sequenced and analysed

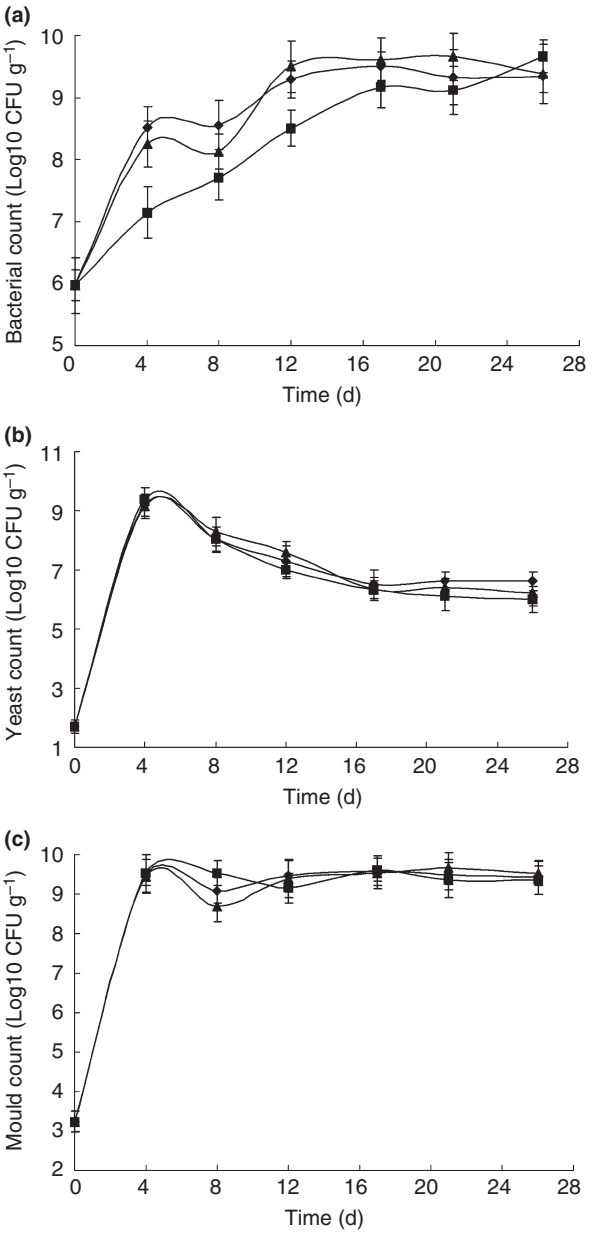


**Figure 3** Microbial successions of bacteria (a), yeast (b) and moulds (c) in *Daqu* during the manufacturing process. GC (♦), HX (■) and HH (▲).

to identify the corresponding micro-organisms with higher than 96% (bacteria) or 98% (fungi) nucleotide sequence identities with sequences in GenBank.

As shown in Fig. 4a, the wide diversity of bacterial species was revealed in primal *Daqu*. Of the 12 bands analysed, as DNA sequences of five bands corresponded to lactic acid bacteria (LAB), LAB constituted one of the dominant bacterial groups in *Daqu*. And a propagation tendency of LAB was observed during the preparation process. The detected LAB species were identical, and the

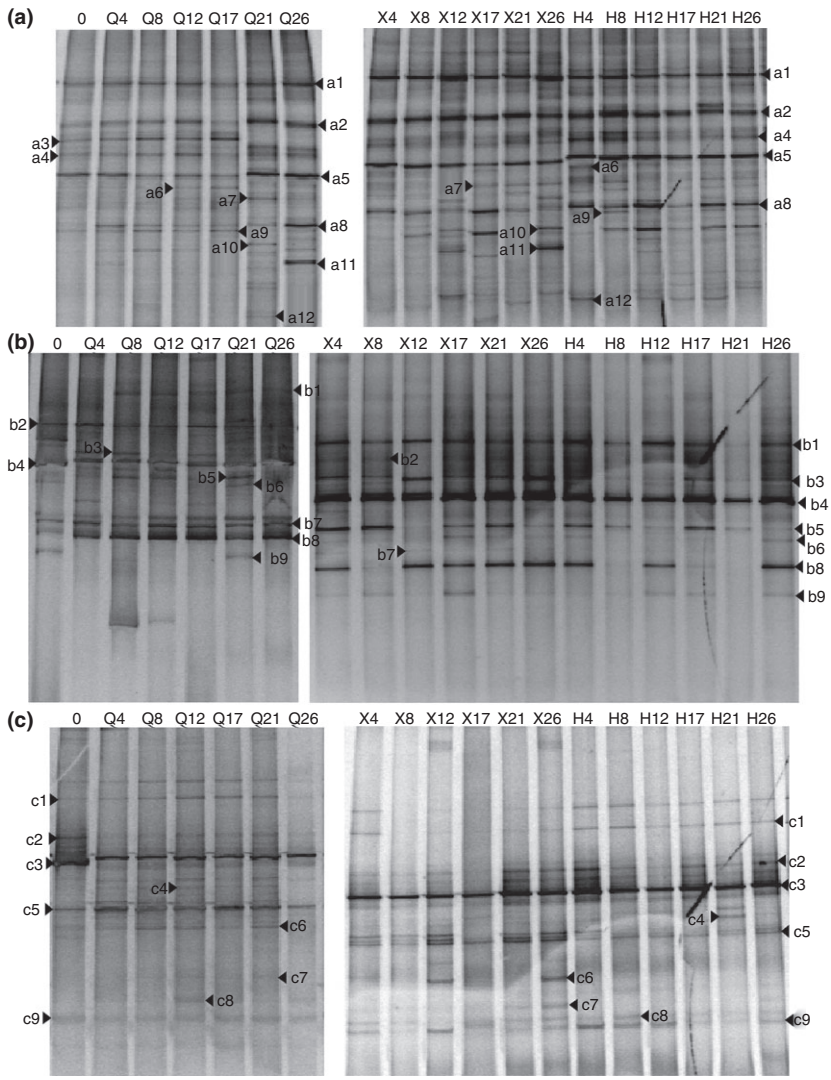


**Figure 4** DGGE analysis of (a) bacteria, (b) fungi and (c) yeast composition in *Daqu*. Q: QC, X: HX, H: HH; Numbers 0, 4, 8, 12, 17, 21 and 26 represent sampling time points. a1: *Weissella cibaria*, a2: *Staphylococcus xylosus*, a3: *Lactobacillus brevis*, a4: *Lactobacillus pontis*, a5: *Lactobacillus panis*, a6: *Lactobacillus crustorum*, a7: *Virgibacillus* sp., a8: *Pseudomonas* sp., a9: *Oceanobacillus* sp., a10: *Thermoactinomyces sanguinis*, a11: *Stenotrophomonas maltophilia* and a12: *Bacillus* sp. b1: *Saccharomyces boulardii*, b2: *Candida tropicalis*, b3: *Rhizopus oryzae*, b4: *Amylomyces rouxii*, b5: *Aspergillus oryzae*, b6: *Aspergillus terreus*, b7/b8: *Absidia blakesleeana* and b9: *Rhizomucor miehei*. c1: *Candia silvae*, c2/c3: *Saccharomycopsis fibuligera*, c4: *Hanseniaspora guilliermondii*, c5: *Pichia anomala*, c6: *Debaryomyces hansenii*, c7: *Issatchenkia orientalis*, c8: *Trichosporon asahii* and c9: *Saccharomyces cerevisiae*.

contents of LAB in three types of *Daqu* changed with the incubation temperature. The results obtained from incubating *Daqu* partly overlap with those from matured *Daqu* (Zhang *et al.* 2014) and difference appeared in detected LAB species and their relative proportion, which was caused by the analysis methods used and regrowth of micro-organisms during the maturing process in the ventilation environment.

In the completed three types of *Daqu*, *Weissella cibaria* (band a1), *Staphylococcus xylosus* (band a2) and *Lactobacillus panis* (band a5) became the main species. The optimal temperature for *Thermoactinomyces* (band a10) growth was about 50–55°C (Yoon *et al.* 2005), and its relative content was higher in HX and HH. The optimum culture temperature of *Stenotrophomonas maltophilia* (band a11) was about 30°C, and it can be cultured between 35 and 40°C (Zhang *et al.* 2008; Nyč and Matějková 2010). The brightness of corresponding band in HH was very weak.

Although *Bacillus* was abundant in *Daqu* (Zheng *et al.* 2013; Zhang *et al.* 2014), only one *Bacillus* sp. was detected in DGGE gel. *Bacillus* species could not be

differentiated from the microbial mixture based on the migration of PCR products using P2/P3 primers by DGGE analysis (Kim *et al.* 2009).

Yeast and moulds were the main eukaryotes in *Daqu* and first analysed as a whole eukaryote group (Fig. 4b). The sequences of the excised DGGE bands were closely related to six species of moulds and one species of yeast. Fungal diversity in QC was higher than those in HH and HX, due to the lower incubation temperature of QC. The relative concentrations of *Rhizopus oryzae* (band b3) and *Amylomyces rouxii* (band b4) were the highest in HX. Relative contents of *Absidia blakesleeana* (band b7/b8) and *Rhizomucor miehei* (band b9) were the highest in QC and HH, respectively. Of the detected fungi, *Rz. oryzae* and *Ab. blakesleeana* belong to thermotolerant fungi (Mouchacca 2007) and *Rh. miehei* is a thermophilic mould (Morgenstern *et al.* 2012). Therefore, the different incubation temperature led to the formation of special heat-tolerant fungal composition in *Daqu*.

Yeast diversity was underestimated using primers of NL1-GC/LS2 and analysed again targeting 26S rRNA gene (Fig. 4c). Most yeast are mesophilic. Among eight species detected, only *Saccharomycopsis fibuligera* (band c2/c3) and *Pichia anomala* (band c5) were predominant in all of *Daqu*. Other yeast were killed by high temperature, and some regrew after 17 days of fermentation with the temperature near to 30°C.

DGGE gels were further analysed using PCA, which produced a graphical representation of samples along with two axes of reference that explain a higher percentage of total variability. Figure 5 reveals the clear discrimination between microbial compositions in *Daqu*. In all of PCA data sets, the first two principal components (PC1 and PC2) explained more than 60% of the total variance. All of bacteria, yeast and fungi community structure in *Daqu* were divided into three groups corresponding to QC, HX and HH.

In conclusion, it is possible that temperature plays a decisive role in the formation of microbial community structure. Even though raw materials, pretreatment, manufacturing environment and microbial source were identical, incubation temperature affected growth and death of micro-organisms, altered the amount of micro-organisms, especially heat-sensitive and heat-tolerant species, and formed the especial microbial composition in the completed *Daqu*. These results need validation in the manufacturing process of low-temperature *Daqu* in the other distilleries, even that of medium-temperature *Daqu* and high-temperature *Daqu*. In the following fermentation, using a combination of three types of *Daqu* as starter culture would enrich the microbial community and increase the diversity of flavour compounds in the brewing system, consequently improving the quality of light aroma style liquor.

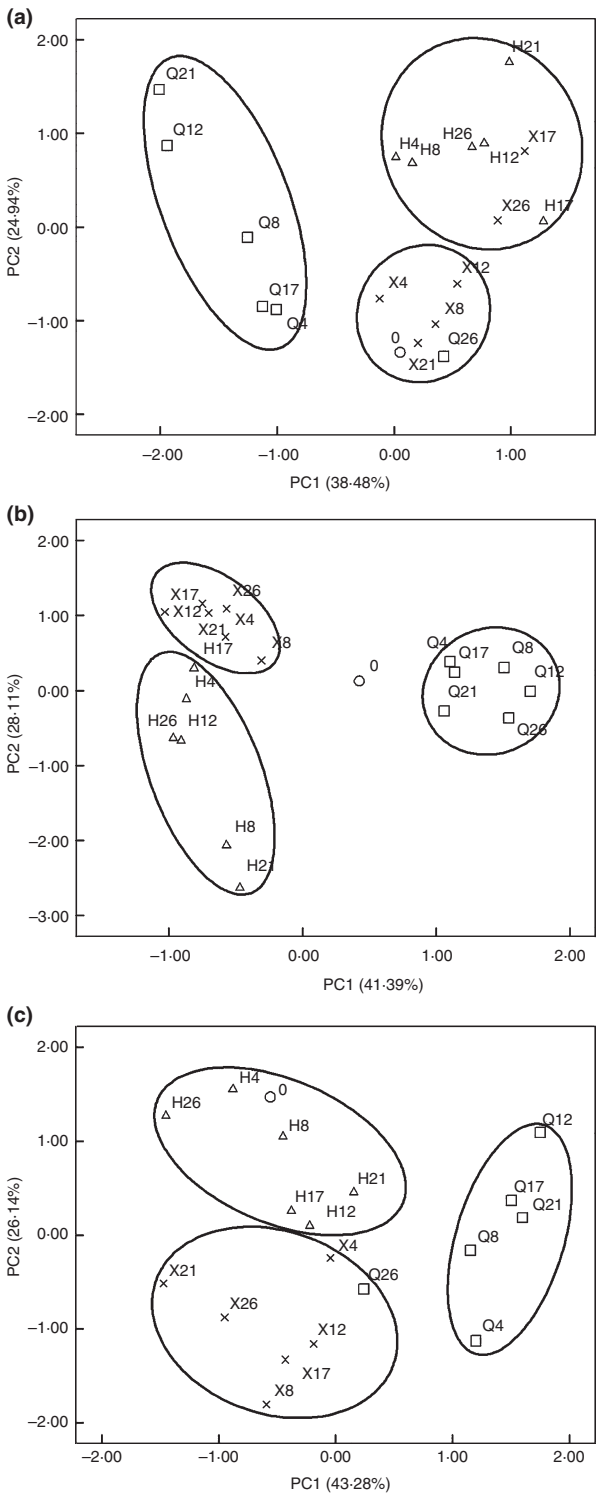


**Figure 5** PCA of DGGE profiles of bacteria (a), fungi (b) and yeast (c). Q: QC, X: HX, H: HH; Numbers 0, 4, 8, 12, 17, 21 and 26 represent sampling time points.

## Materials and methods

### Sampling

*Daqu* was collected from the Shanxi Xinghuacun Fenjiu Distillery Co. Ltd. (Fenyang, Shanxi, China). The incubation phase of the *Daqu* preparation process contained five stages. Sampling of QC, HX and HH was taken at 0, 4, 8, 12, 17, 21 and 26 days at the typical time point of every stage and the initial and last day. At every sampling time, three bricks of *Daqu* were randomly collected, milled and mixed as one sample. Samples of *Daqu* were transferred into sterile bags. Half of the samples were stored at −70°C to analyse microbial community compositions, and the rest were kept at 4°C for enumerations of bacteria, yeast and moulds. Temperatures were recorded by a portable infrared thermometer (WFHX-68, Shanghai, China) at the time of sampling in the middle of the *Daqu* pile.

### Microbiological analysis of culturable microbiota

Ten g of *Daqu* was suspended into 95 ml sterile physiological solution (0·85% NaCl) and homogenized in the incubator at 200 rev $min^{-1}$, 4°C for 30 min and serially diluted (1 : 10). 0·1 ml volumes of suitable dilutions were spread in triplicate on plates of the following agars: (i) beef extract peptone medium (3 g beef extract, 10 g peptone and 5 g NaCl per litre) incubated at 37°C for 24 h for the enumeration of aerobic bacteria, (ii) Dichloranrose bengal medium (Ourchem, Shanghai, China) incubated at 30°C for 4 days for enumerating moulds, and (iii) YPD medium (Sangon Biotech, Shanghai, China) incubated at 30°C for 48 h for the enumeration of yeast. Results were calculated as the means of log10 CFU $g^{-1}$ for three determinations.

### PCR-Denaturing gradient gel electrophoresis (DGGE) analysis

DNA was extracted from *Daqu* samples according to the methods described by Wang *et al.* (2008). Three primer pairs of P2/P3 (Muyzer *et al.* 1993), NL1-GC/LS2 (Cocolin *et al.* 2002) and NS1/GCfung (May *et al.* 2001) were used to amplify bacterial 16S rRNA V3 region gene, yeast 26S rRNA D1/D2 region gene and fungal 18S rRNA gene. Reactions were performed in a C1000 thermal cycler (Bio-Rad, Hercules, CA), and PCR products were subjected to DGGE analysis using DCode Universal Mutation Detection System (Bio-Rad).

Samples were applied to 8% (w/v) polyacrylamide gels in 1 × TAE buffer [20 mmol $l^{-1}$ Tris, 10 mmol $l^{-1}$ acetate, 0·5 mmol $l^{-1}$ EDTA (pH 8·0)]. Optimal separation was achieved with a 30–50% urea–formamide denaturing gradient [100% corresponding to 7 mol $l^{-1}$ urea and 40% (v/v) formamide] for bacteria, 10–35% for fungi and 10–50% for yeast. The gels were electrophoresized for 200 min at 100 V and then stained with SYBR green I (Invitrogen, Carlsbad, CA) for 45 min. The gels were photographed with the GelDos 2000 system (Bio-Rad). Principal component analysis (PCA) of DGGE bands was performed using Quantity One (Bio-Rad) and SPSS, version 17.0 (SPSS Inc., Chicago, IL).

The bands of interest were excised from the gels, incubated overnight at 4°C in distilled water and re-amplified using corresponding primers. PCR products were ligated into pMD19-T Easy vector (Takara, Dalian, China). Plasmids DNA were extracted from randomly selected clones, screened for inserts of the expected size and correct DGGE migration properties and sequenced at Sangon Biotech (Shanghai) Co., Ltd. (China). Homology searches were performed in GenBank to determine the closest known relatives of the partial rRNA sequences obtained.

## Acknowledgements

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## Conflict of interest

The authors have no conflict of interests.

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