Food Microbiology 76 (2018) 11–20

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Characterization of bacteria and yeasts isolated from traditional fermentation starter (*Fen-Daqu*) through a ^{1}H NMR-based metabolomics approach

Rui-Yao Li [a, 1], Xiao-Wei Zheng [b, e, 1], Xin Zhang [c], Zheng Yan [d], Xiao-Yong Wang [c], Bei-Zhong Han [a, *]

[a] Beijing Laboratory of Food Quality and Safety, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100083, China
[b] Biotechnology Center, Nutrition & Health Research Institute, COFCO Corporation, Beijing Key Laboratory of Nutrition & Health and Food Safety, Beijing Engineering Laboratory for Geriatric Nutrition Food Research, Beijing, China
[c] Shanxi Xinghuacun Fenjiu Distillery Co. Ltd., Fenyang, 032205, China
[d] College of Bioengineering, Beijing Polytechnic, Beijing, 100176, China
[e] Beijing Key Laboratory of Nutrition, Health and Food Safety & Beijing Engineering Laboratory for Geriatric Nutrition Food Research, Nutrition & Health Research Institute, COFCO Corporation, Beijing, 102209, China

ARTICLE INFO

Article history:
Received 13 August 2017
Received in revised form
15 March 2018
Accepted 29 March 2018
Available online 30 March 2018

Keywords:
Fermentation starter
^{1}H NMR
Metabolites
Saccharification
Ethanol
Functionality

ABSTRACT

Daqu is a traditional fermentation starter for the production of *baijiu* and vinegar. It is an important saccharifying and fermenting agent associated with alcoholic fermentation and also a determining factor for the flavour development of these products. Bacterial and yeast isolates from a traditional fermentation starter (*Fen-Daqu*) were examined for their amylolytic activity, ethanol tolerance and metabolite production during sorghum-based laboratory-scale alcoholic fermentation. The selected strains (*Bacillus licheniformis*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Pichia kudriavzevii*, *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae*, and *Saccharomycopsis fibuligera*) were blended in different combinations, omitting one particular strain in each mixture. ^{1}H nuclear magnetic resonance (NMR) spectroscopy coupled with multivariate statistical analysis was used to investigate the influence of the selected strains on the metabolic changes observed under the different laboratory-controlled fermentation conditions. Principal component analysis showed differences in the metabolites produced by different mixtures of pure cultures. *S. cerevisiae* was found to be superior to other species with respect to ethanol production. *S. fibuligera* and *B. licheniformis* converted starch or polysaccharides to soluble sugars. Lactic acid bacteria had high amylolytic and proteolytic activities, thereby contributing to increased saccharification and protein degradation. *W. anomalus* was found to have a positive effect on the flavour of the *Daqu*-derived product. This study highlights the specific functions of *S. cerevisiae*, *S. fibuligera*, *B. licheniformis*, *W. anomalus* and lactic acid bacteria in the production of light-flavour *baijiu* (*fen-jiu*). Our results show that all investigated species deliver an important contribution to the functionality of the fermentation starter *Daqu*.

1. Introduction

Baijiu and vinegar are products that are obtained from cereals such as sorghum and wheat by complex fermentation processes using natural mixed culture starters (i.e., *Daqu*) (Zheng et al., 2011; Zheng and Han, 2016). *Baijiu* and vinegar contain a number of metabolites that either originate directly from the raw materials and ingredients, such as the sorghum and *Daqu,* or are produced during alcoholic fermentation by the consortia of yeasts and bacteria originating from the *Daqu*. Although the constituents of sorghum affect metabolite formation, the vast majority of components found in *baijiu* and vinegar are of microbial origin and produced during fermentation (Li et al., 2014a; Zheng et al., 2014). *Daqu*

* Corresponding author. College of Food Science & Nutritional Engineering, China Agricultural University, P.O. BOX 398, No. 17 Qinghua East Road, Beijing, 100083, China.
E-mail addresses: lierking2008@126.com (R.-Y. Li), xiaowei.zheng@wur.nl (X.-W. Zheng), bioyanzheng@126.com (Z. Yan), hbz@cau.edu.cn (B.-Z. Han).
[1] Rui-Yao Li and Xiao-Wei Zheng contribute equally to this work.

https://doi.org/10.1016/j.fm.2018.03.015
0740-0020/

contains a diversity of microorganisms, and has significant effects on ethanol production and flavour development during alcoholic fermentations (Zheng et al., 2012). Predominant groups of bacteria and yeasts in *Fen-Daqu* (viz., *Bacillus licheniformis, Pichia kudriavzevii*, *Saccharomycopsis fibuligera, Wickerhamomyces anomalus*, and lactic acid bacteria [LAB]) were isolated and identified previously (Zheng et al., 2012, 2014). However, not much is known about the specific contributions of these microorganisms to the of *baijiu* or vinegar production. Therefore, we characterized the effect of these microorganisms on saccharification, ethanol production, and flavour production during lab-scale alcoholic fermentation studies. Microbes isolated from mixed microbial populations obtained from traditional fermented foods are shown to exhibit strain specific metabolic activities (Nyanga et al., 2013; Smid and Kleerebezem, 2014). Therefore, we performed an extensive screening exercise with the objective to select candidate species for use in starter cultures.

^{1}H NMR-based metabolomics coupled with principal component analysis (PCA) was used to evaluate the performance of selected bacterial and yeast strains isolated from *Fen-Daqu* during alcoholic fermentation.

2. Materials and methods

2.1. Screening of bacteria and yeast isolates for sorghum fermentation

2.1.1. Cultivation of strains

A total of 161 microbial cultures, comprising 72 isolates of *B. licheniformis*, 46 isolates of *S. fibuligera*, 9 isolates of *P. kudriavzevii*, 26 isolates of *W. anomalus*, 1 isolate of *Saccharomyces cerevisiae*, and 7 isolates of LAB (*Pediococcus pentosaceus* and *Lactobacillus plantarum*), were previously isolated from *Daqu* (Zheng et al., 2012). Eight reference *S. cerevisiae* strains were obtained from the College of Food Science and Nutritional Engineering of China Agricultural University. Yeasts were grown on malt extract agar (MEA; Oxoid CM0059), bacilli were grown on plate count agar (PCA; Oxoid CM035), and LAB were grown on MRSA (Oxoid CM0361). All stocks were stored in 30% glycerol at −80 °C.

2.1.2. Preparation of inoculum

Cultures were incubated for 2 d at 30 °C in 10 ml nutrient broth (NB; Oxoid CM0001) for bacteria or YPD broth (Sigma Y1375) for yeast. Suspensions of 10^8 cells/ml were made in sterile peptone physiological salt solution (0.85%) (Oxoid, BO0471) as confirmed by microscopic counts.

2.1.3. Starch degradation assay

An aliquot (1 μl) of the inoculum was transferred to the center of a starch agar plate and incubated at 30 °C for 2 d. Starch degradation was visualized by flooding the plate with a 0.25% iodine solution. The diameter of the colony and surrounding halo were recorded, and the ratio of these diameters (D_h/D_c) was calculated as a measure of starch degradation (Jančič et al., 2015).

2.1.4. Enzyme activity assays

An aliquot (1 ml) of culture was grown in 10 ml of growth medium (NB for bacilli, MRS broth for LAB, and malt extract broth [MEB] for yeast) with 0.1 g of crushed sorghum. The crude enzyme was obtained by centrifugation at 10,000 × *g* for 10 min at 4 °C (Tabassum et al., 2014). Each time the obtained supernatant was used to assay for amyloglucosidase and α-amylase activities, respectively.

(1) Amyloglucosidase assay

Amyloglucosidase was assayed by using a test kit (Megazyme, RAMGR3) with p-nitrophenyl-β-maltoside as a substrate, according to the manufacturer's instructions.

(2) α-Amylase assay

α-Amylase activity was assayed by using the EnzyChrom α-Amylase Assay Kit (Bioassay system, Hayward, USA) according to the manufacturer's instructions.

2.1.5. Alcohol tolerance test

All strains were tested for their ethanol tolerance by using the spot test according to Kim et al. (2011). The strains were grown in MEB (for yeast) and NB broth (for bacteria) to an OD_{600} of 1, and then diluted ten-fold with a sterile physiological salt solution (0.85%, w/v). Aliquots (1 μl) of each suspension were spotted onto MEA or PCA plates containing 0%, 4%, 8%, or 12% (v/v) ethanol, which were incubated at 30 °C for 2 d. Ethanol tolerance was calculated based on colony size (diameter). Each strain was plated in duplicate.

2.2. Fermentation tests

2.2.1. Preparation of inoculum

Based on the starch degradation and alcohol tolerance screening results mentioned above, seven strains (*B. licheniformis* 128, *P. pentosaceus* 6, *L. plantarum* 1, *P. kudriavzevii* 12, *W. anomalus* 29, *S. cerevisiae* 1, and *S. fibuligera* 4) were selected for lab-scale fermentation. Each strain was grown in 10 ml of NB (bacteria) or MEB (yeasts) at 30 °C for 2 d. A 1-ml aliquot of the culture was then centrifuged at 2000 × *g* for 10–15 min. The cell pellets were suspended in a sterile physiological salt solution (8.5 g/l) and adjusted to a density of 10^7 CFU/ml for yeast and 10^8 CFU/ml for bacteria.

2.2.2. Preparation of sorghum mixture

Aliquots of sorghum crude powder (100 g; obtained from Xinghuacun *Fen-jiu* (Zheng and Han, 2016) Group, Shanxi province, China in Jan. 2013) were mixed with 80 ml of hot water (80 °C) and soaked for 24 h. After soaking, the mixture was steamed in an autoclave for 30–40 min at 100 °C. The obtained sorghum paste was mixed with sterile cold water (30 g/100 g paste; 18–20 °C), and then cooled to room temperature.

2.2.3. Laboratory-scale fermentation

Hundred g of the sorghum mixture was placed into a sterile 250-ml conical flask and then closed with a water lock. Nine independent alcoholic fermentations were carried out as shown in Table 1. According to the experimental design, 1 ml of total microbial suspension containing of yeast suspension (10^7 CFU/ml) and bacteria suspension (10^8 CFU/ml), or 10 g of powdered *Daqu* were used (Table 1). The fermentation flasks were incubated at 25 °C for 28 d. Samples (approximately 30 g) were taken on 0, 14, and 28 d. Each fermentation (flask) was performed in triplicate.

The combination matrix of different cultures is presented in Table 1. The selected strains were mixed together in a series of cultures, each with 1 strain omitted, except for fermentation M, which contained all the candidate strains. The culture mixtures were added to sorghum, and the alcoholic fermentation process of *fen-jiu* (see materials and methods) was followed. One control fermentation with *Fen-Daqu* was used in this study.

2.3. Physicochemical analyses

2.3.1. pH measurement

The pH of the fermentations was measured with a pin electrode

Table 1
Experimental design of fermentation trials with different strain mixtures.

Mix	S.c-1	S.f-4	W-29	P-12	B-128	L.p-1	P.p-6	*Daqu*
M	X	X	X	X	X	X	X	
F1		X	X	X	X	X	X	
F2	X		X	X	X	X	X	
F3	X	X		X	X	X	X	
F4	X	X	X		X	X	X	
F5	X	X	X	X		X	X	
F6	X	X	X	X	X		X	
F7	X	X	X	X	X	X		
D								X

NoteB = Bacillus licheniformisP.p = Pediococcus pentosaceus; L.p = Lactobacillus plantarum; P = Pichia kudriavzevii; W = Wickerhamomyces anomalus; S.c = Saccharomyces cerevisiae; S.f = Saccharomycopsis fibuligera. "Cross" indicates that the strain was added to the fermentation.

and a pH meter (Thermo) inserted directly into each sample suspension (1 g/10 ml). Three independent measurements were done on each sample.

2.3.2. Determination of moisture content

The moisture content of the samples was determined using a standard oven drying method at 105 °C until constant weight was reached. The determinations were conducted in triplicate and the mean values calculated.

2.4. Chemical analysis

2.4.1. Extraction of polar compounds

Polar compounds were extracted from the fermented materials according to the method of Le et al. (2011) with a minor modification. Briefly, 300 mg of sample, (instead of 100 mg to increase the concentration of extract) was transferred to a centrifuge tube containing 1.5 ml of cold Milli-Q water. The solution was mixed and vortexed at 2500 oscillations/min for 1 min using a Biospec Beadbeater (Mini Beadbeater-8; Biospec, Bartlesville, USA) without beads. The tube was then incubated on ice for 10 min, and centrifuged at 16,060 × *g* for 10 min at 4 °C. The centrifugation was repeated twice until the supernatant became clear. The clear supernatant was then transferred to a new tube and stored at −80 °C until analysis. Each extraction was performed in triplicate.

2.4.2. NMR

The aqueous extracts for NMR measurements were prepared as reported previously (Wu et al., 2009). A 600 μl aliquot of each sample was transferred into a 5-mm NMR tube. All ^{1}H NMR spectra were measured at 300 K using an AVANCE NMR spectrometer (proton frequency = 600.13 MHz, 14.1 T; Bruker, Billerica, Germany) with a cryogenic NMR probe. The ^{1}H NMR experiments were performed using the following conditions: NOESYGPPRR1D pulse sequence; relaxation delay, 4 s; mixing time (for NOESY), 1 s; acquisition time, 2.28 s; number of steady states transients (dummy scans), 4; gradient pulse time, 1 ms; solvent suppression, presaturation with spoil gradient; spectral width, 7184 Hz; and time domain size, 32 k. The compounds were identified and quantified with Chenomx software (version 7.6; Chenomx, Edmonton, Canada) with reference to the internal standard TSP. Each ^{1}H NMR spectrum was equally divided into 242 fragments with width of 0.04 ppm. Spectra both with a range of 0.00–10.00 ppm and exception of residual water resonance (4.5–4.8 ppm), were divided into 0.04 ppm wide bins, followed by importing the achieved integral values into Microsoft® Excel (Microsoft Corporation, Redmond, WA, USA).

2.5. Statistical analysis

The plate counts of the triplicate experiments were log transformed, and the averages and standard errors of the mean were calculated. Statistical analyses were performed using IBM-SPSS V19.0 (IBM, SPSS Statistics; NY, USA). One-way ANOVA with Duncan's test were used to determine the significance of differences in physical data (i.e., pH and moisture). NMR data were analyzed by PCA using AMIX software (version 3.7.10; BrukerBioSpin, Rheinstetten, Germany). Before performing the PCA, the NMR spectra ($\delta = 0.70–9.20$) were segmented into 0.04-ppm bins. The water region ($\delta = 4.4–5.3$) and imidazole regions ($\delta = 7.35–7.50$ and $\delta = 8.4–8.6$) were excluded from the analysis. All spectral data were first scaled to the total intensity of the corresponding spectrum using AMIX software so that the relative concentration of each compound could be expressed as the relative intensity of each spectrum. The output from the PCA analysis consisted of score plots, which provided an indication of the differentiation of the classes in terms of metabolome similarity, and loading plots, which provided an indication as to which NMR spectral regions were important with respect to the classification obtained in the score plots.

3. Results

3.1. Screening of bacterial and yeast isolates for starch degrading ability

A total of 79 bacterial and 90 yeast isolates were tested for their starch degrading abilities. The amyloglucosidase activity of *B. licheniformis* was only approximately 0.03 U/g (dw), whereas some *P. pentosaceus* and *L. plantarum* showed activities as high as 0.1–0.3 U/g (dw) (see Fig. 1). *P. pentosaceus* 6 (P.p-6) and *L. plantarum* 1 (L.p-1) also showed high starch degrading abilities with D_h/D_c value of 3.3 and 3.6, respectively. Of the *B. licheniformis* isolates, strain B-128 showed the highest starch degradation (D_h/D_c: 3.1). In addition, this strain also showed a higher ability to produce amyloglucosidase as compared to the other *B. licheniformis* strains.

Of the four yeast species studied, *S. fibuligera* had generally a higher starch degrading activity than the other 3 species (*P. kudriavzevii*, *S. cerevisiae* and *W. anomalus*), see Fig. 2. *W. anomalus* (W-29) showed high starch degrading ability (D_h/D_c: 1.4) and high α-amylase activity (0.144 U/g).

More than 90% of the *S. fibuligera* strains were able to produce amyloglucosidase and α-amylase. The highest starch degrading yeast strain was *S. fibuligera* strain S.f-26. This strain not only had the highest ratio of 2.1, but also had high α-amylase and amyloglucosidase activities, with values of 0.143 U/g and 0.064 U/g, respectively. Except for strain *S. cerevisiae* 1, the other *S. cerevisiae* strains were isolated from traditionally fermented red wine grapes. In general, amyloglucosidase activity had less discriminating effect compared to the other two factors including α-amylase activity and D_h/D_c (Duncan's test, data not shown).

3.2. Screening bacterial and yeast isolates for ethanol tolerance

The ethanol tolerance of bacterial and yeast strains is shown in Tables 2 and 3, respectively. Of the bacteria, *P. pentosaceus* 2 and 6 (P.p-2 and P.p-6) were able to grow up to 12% (v/v) alcohol, whereas *L. plantarum* was able to tolerate up to 8% alcohol. Only a few *B. licheniformis* strains (B-9, B-41, B-43, B-49, B-104, B-106, and B-121) were able to grow with 12% alcohol; however, most *B. licheniformis* strains only showed growth on 4% alcohol.

One *P. kudriavzevii* strain (P-1) was able to tolerate up to 12%

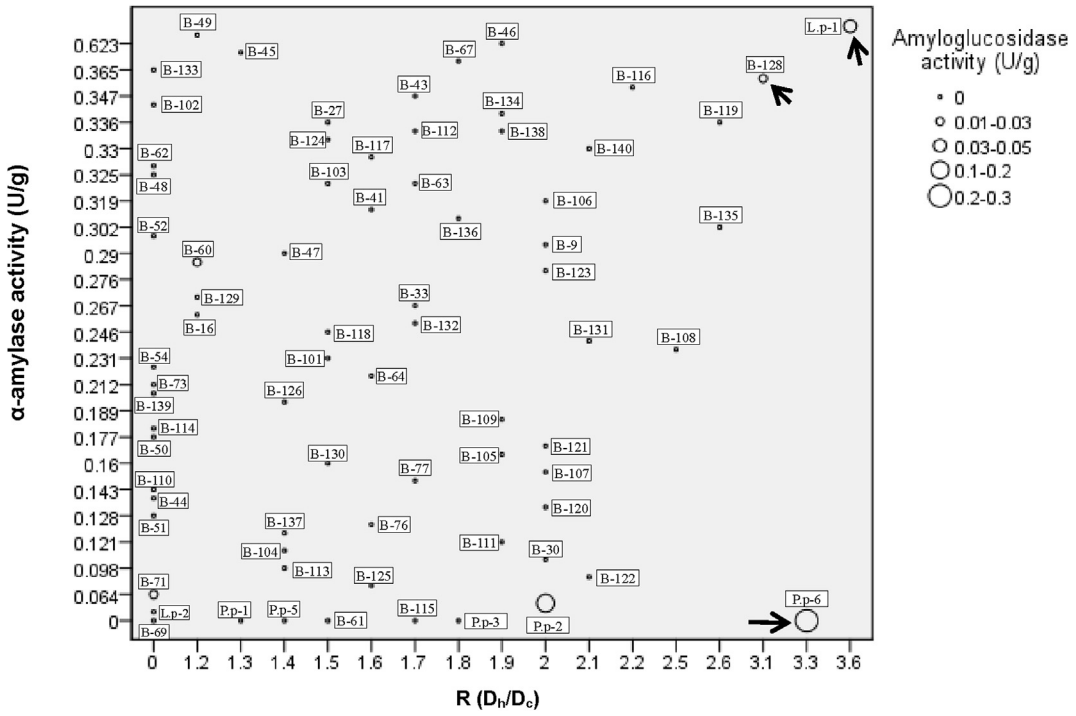

Fig. 1. Bubble plot diagram of starch degradation (X-axis), α-amylase activity (Y-axis) and amyloglucosidase activity (bubble size, see legend top right) of bacterial strains. Note: B = *Bacillus licheniformis*; P.p = *Pediococcus pentosaceus*; L.p = *Lactobacillus plantarum*; D_h = The diameter of halo; D_c = the diameter of colony. Arrows indicate the selected strains.

alcohol, and the other strains showed variable tolerances of 4% and 8% alcohol (Table 3). None of the *W. anomalus* strains showed growth in 12% alcohol, and only 9 of these were able to tolerate 8% ethanol. One *S. cerevisiae* strain (S.c-1) was able to tolerate up to

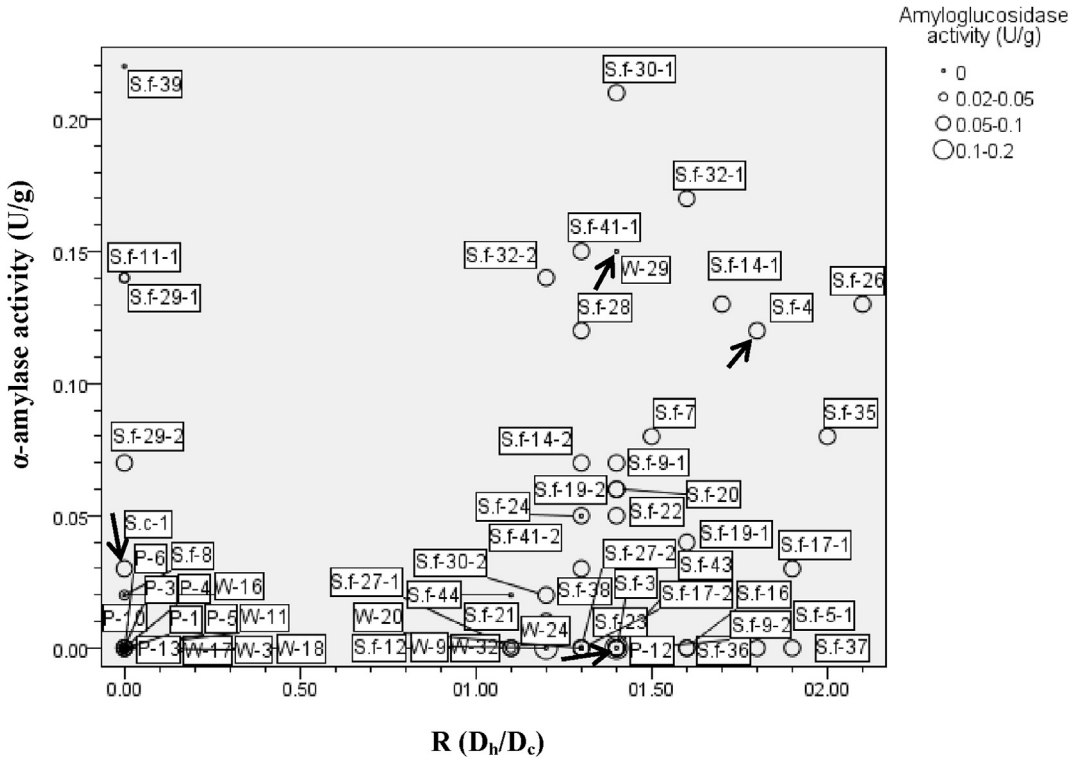

Fig. 2. Bubble plot diagram of starch degradation of (X-axis), α-amylase activity (Y-axis) and amyloglucosidase activity (bubble size, see legend top right) yeast strains. Note: P = *Pichia kudriavzevii*; W = *Wickerhamomyces anomalus*; S.c = *Saccharomyces cerevisiae*; S.f = *Saccharomycopsis fibuligera*. Twenty-three strains (P-6, P-10, P-12, P-13, W-3, W-4, W-5, W-8, W-10, W-11, W-14, W-15, W-17, W-18, W-19, W-21, W-22, W-27, W-30, W-36, W-43, S.c-SC8, Sc-YS7) were not included in the figure since no starch degradation activities were observed. Arrows indicate the selected strains.

Table 2
Alcohol tolerance test of bacterial isolates.

Bacteria	Growth on medium* containing ethanol		
	4% ethanol	8% ethanol	12% ethanol
B. licheniformis (9,41,43,49,121)	+	+	+
B. licheniformis (104,106)	+	+	w
B. licheniformis (42,63,71,76,108,112,118,120,134,138)	+	+	–
B. licheniformis (60,69,123)	+	w	w
B. licheniformis (47,65,122,128,131)	+	w	–
B. licheniformis(109)	+	v	–
B. licheniformis (16,27,30,33,44,45,48,50,51,52,54,55,62,64,66,67,73,77,101,102,103,105,107,110,111,114,115,116,117,119,124,125,126,127,129,130,132,133,135,136,137,139,140)	+	–	–
B. licheniformis (46)	–	–	–
P. pentosaceus (2,6)	+	+	+
P. pentosaceus (5)	+	+	–
P. pentosaceus (1,3)	+	–	–
L. plantarum (2)	+	+	v
L. plantarum (1)	+	–	–

Notes: + = positive, w = weak, - = negative, v = variable. A control of media without alcohol showed growth for all bacterial strains. *for *B. licheniformis*, Plate count agar was used; for lactic acid bacteria, MRSA was used.

12% alcohol. Half of the *S. fibuligera* strains were able to tolerate 8% alcohol and of these, 4 strains (S.f-4, S.f-6, S.f-9, and S.f-43) were able to tolerate 12% alcohol.

3.3. Design of fermentation trials by mixing pure bacterial and yeast cultures

First, high starch degrading ability was used as a criteria to select candidate bacterial and yeast strains (except *S. cerevisiae*) for fermentation trails. Simultaneously, the ethanol tolerance of the selected strains was compared. *B. licheniformis* 128 and *P. pentosaceus* 6 were selected based on the above mentioned criteria. Although strain *L. plantarum* 1 had a low ethanol tolerance, this species is frequently encountered in *baijiu* fermentations (Li et al., 2011); therefore, we also included this strain in the fermentation trials. *W. anomalus* 29 and *P. kudriavzevii* 12 were selected for their high starch degradation ability; *S. fibuligera* 4 and *S. fibuligera* 26 were similar in starch degradation ability, *S. fibuligera* 4 instead of *S. fibuligera* 26 was selected for its high alcohol tolerance.

3.4. Monitoring pH and moisture content during fermentation

Fermentation trial D (containing *Daqu*) showed a lower initial pH value (pH 6.1) as compared to the other fermentation trials (Fig. 3A). After 28 days of incubation, the pH values of all the fermentations reached the same level as fermentation D, except for F1 and F7 that showed less acidification.

Fig. 3B shows the changes in moisture content in all trials during alcoholic fermentation. A similar level in moisture content was detected in most of trials throughout the fermentation process, with trial D showing significantly higher values.

3.5. Characteristics of the 9 different fermentation trials

The comparison of different trials with respect to metabolite composition was performed by PCA. All the trials were individually compared to trial M based on their metabolites composition using pairwise PCA score plots (Fig. 4). PC1 scale illustrates the effects of the changes of condition on the metabolites profile. The PC2 scale gives the differences between the triplicates and is enlarged over the PC2 scale but generally is of much minor magnitude than the PC1 effect. To identify the metabolites responsible for the variations in the PCA score plots in the different fermentation trials, loading plots of PC1 were generated (Fig. 4I–P). The upper sections of the loading plots represent the concentrations of metabolites that were higher in fermentations F1–F7 and D, whereas the lower sections revealed metabolite concentrations that were higher in fermentation M. Lactate and acetate are two important microbial metabolites that occurred during alcoholic fermentations. It shows that the higher concentrations of acetate and lower concentrations of lactate are detected in fermentation 1 (without *S. cerevisiae*), 3 (without *W. anomalus*), 5 (without *B. licheniformis*), and 7 (without *P. pentosaceus*), whereas opposite trend at lower acetate and higher lactate level were detected in fermentation 2 (without *S. fibuligera*) and 4 (without *P. kudriavzevii*) when compared to the levels in fermentation M. Fig. 4N shows that the concentrations of lactate and acetate were all higher in fermentation 6 (without *L. plantarum*) than in fermentation M, whereas the concentrations of alanine and butyrate were higher in the latter fermentation. Amino acids such as alanine and threonine were detected with higher concentration in fermentation M when compared to fermentation 2 (Fig. 4J). Ethanol was detected in higher concentration in fermentation 6, 7 and D (Fig. 4N, O and P), with significant higher level in fermentation D. Fig. 4P shows the differentiation of fermentation M and fermentation D (with *Daqu*). It shows that the

Table 3
Alcohol tolerance of yeast isolates.

Yeasts	Growth on MEA containing ethanol		
	4% ethanol	8% ethanol	12% ethanol
P. kudriavzevii (1)	+	+	+
P. kudriavzevii (4,6,13,14)	+	+	−
P. kudriavzevii (10)	+	−	w
P. kudriavzevii (3,5,12)	+	−	−
W. anomalus (8,10,11,15,25,27,29)	+	+	−
W. anomalus (5,28)	+	w	−
W. anomalus (23)	+	v	−
W. anomalus (3,4,7,9,14,16,17,18,19,20,21,22,24,30,32,36)	+	−	−
S. cerevisiae (1)	+	+	+
S. cerevisiae (NJSC5)	+	+	v
S. cerevisiae (GM6,NJSC5,YS7)	+	+	−
S. cerevisiae (CC18,SC8,SS2,YS8)	+	−	−
S. fibuligera (4,9)	+	+	+
S. fibuligera (6,43)	+	+	v
S. fibuligera (1,5,8,10,11,13,17,22,25,31,33,36,41,42,44)	+	+	−
S. fibuligera (16,28,34,37,38)	+	w	−
S. fibuligera (27)	+	v	−
S. fibuligera (2,3,7,12,14,15,18,19,20,21,23,24,26,29,30,32,35,40,45,46)	+	−	−
S. fibuligera (39)	v	−	−

Notes: + = positive, w = weak, - = negative, v = variable. A control of media containing no alcohol showed growth for all the yeast strains.

concentrations of lactate, butyrate and acetate were higher in fermentation M.

4. Discussion and conclusions

4.1. Screening bacterial and yeast strains for sorghum fermentation

Bacillus and LAB are dominant members of the microbial community of *Daqu* and thus thought to be important for the functionality of the starter (Liu et al., 2012; Zheng et al., 2012). In our study, *B. licheniformis* isolates showed starch degrading abilities with D_h/D_c values in the range of 0–3.1, α-amylase activities in the range of 0–0.598 U/g (dw), and amyloglucosidase activities at levels below 0.03 U/g (dw) (Fig. 1). Some *L. plantarum* and *P. pentosaceus* strains (L.p-1 and P.p-6) showed higher starch degrading activities than *B. licheniformis*. Generally, *B. licheniformis*, *L. plantarum* and *P. pentosaceus* showed a little bit higher starch degrading abilities than yeast isolates, this suggests that these three species are the important starch degraders in the microbial community of *Daqu*.

Only five *B. licheniformis* strains (B-9, B-41, B-43, B-49, and B-121), one *L. plantarum* strain (L.p-2), and two *P. pentosaceus* strains (P.p-2 and P.p-6) were able to tolerate 12% ethanol. Several factors are known to be involved in the ethanol tolerance of bacteria, including ethanol-induced changes in plasma membrane composition and inactivation of cytosolic enzymes (e.g., ATPase and glycolytic enzymes) (Huffer et al., 2011). Some *B. licheniformis* strains (B-9, B-41, B-43, B-49, and B-121) showed a high ethanol tolerance possibly related with the formation of cell macro-fibers and structured filamentous growth when exposed to ethanol stress (Torres et al., 2005). *L. plantarum* and *P. pentosaceus* strains are known to be ethanol tolerant (Liu and Qureshi, 2009), due to solvent induced changes in the membrane lipid composition.

S. fibuligera has been reported as the only yeast species that is present in all different types of *Daqu* (Wang et al., 2011b). In several studies, this yeast was considered as the major amylolytic yeast in indigenous food fermentations (Chen et al., 2010; Chi et al., 2009). This is consistent with our results, as most *S. fibuligera* strains tested showed good starch degrading abilities (Fig. 2).

4.2. Contributions of selected strains during alcoholic fermentation

4.2.1. Saccharomyces cerevisiae

S. cerevisiae is probably the most important yeast species during alcoholic fermentation, as it is able to ferment glucose, sucrose, maltose and galactose to ethanol (Sun, 2014). This was confirmed by our results (Fig. 4I and Table S1) showing that the concentration of ethanol was significantly higher in fermentation M than in fermentation 1 (without *S. cerevisiae*). *S. cerevisiae* produced ethanol more efficiently than any other tested species as the ethanol content (12 mmol/g) in fermentation 1 was the lowest among all fermentation trials, except for Fermentation 5 (without *B. licheniformis*). The presence of *S. cerevisiae* was found to be associated with a decrease in pH (Fig. 3A). Two factors could lead to this change: (1) alcoholic fermentation driven by *S. cerevisiae* is associated with the production of organic acids such as citric acids (Acourene and Ammouche, 2012); (2) *S. cerevisiae* may have a positive effect on the production of lactic acid by LAB (Gül et al., 2005). This synergetic effect can explain the higher concentration of lactic acid (17 mmol/g) in fermentation M if compared to fermentation 1 (0.3 mmol/g) (Table S1).

4.2.2. Saccharomycopsis fibuligera

S. fibuligera is found in starchy substrates worldwide, and is the major amylolytic yeast in indigenous food fermentations involving cereals, such as rice and sorghum (Nie et al., 2013; Saelim et al., 2008). The major contribution of *S. fibuligera* during alcoholic fermentation appears to be the degradation of starch or polysaccharides to small, fermentable molecular sugars, such as maltose, maltotriose, and dextrin, that can subsequently be hydrolyzed to glucose. *S. fibuligera* secretes almost exclusively α-amylase and glucoamylase (Ismaya et al., 2012), and this explains the high α-amylase activity of most tested *S. fibuligera* strains (Fig. 2). Although *S. fibuligera* can produce a large amount of amylases that hydrolyze starch into glucose, it cannot ferment glucose into ethanol. *S. cerevisiae* on the other hand, is unable to convert starch to glucose. Therefore, a mixed culture of *S. cerevisiae* and *S. fibuligera* could increase the production of ethanol, which is suggested by the higher ethanol concentration found in fermentation M than in fermentation 2 (without *S. fibuligera*). This effect

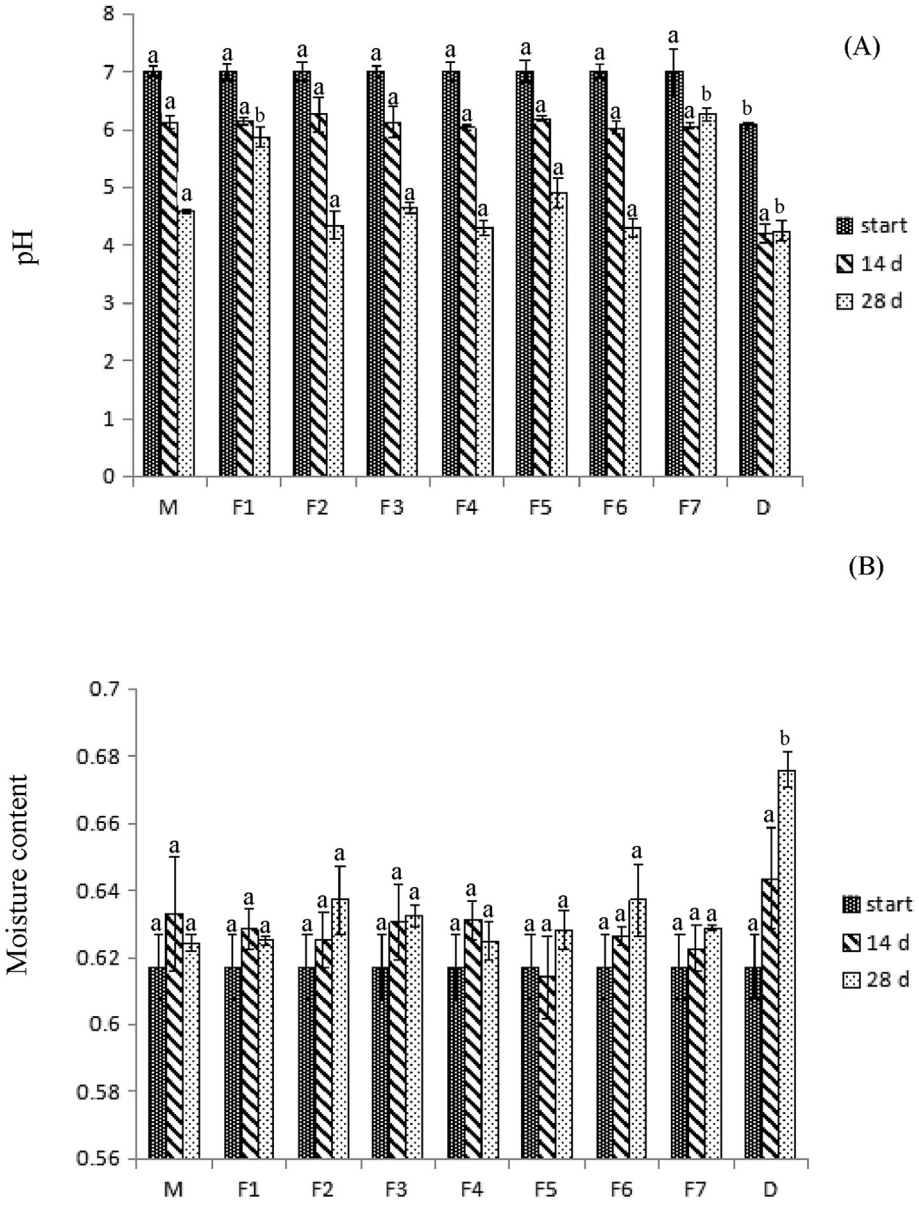

Fig. 3. Changes of pH and moisture content in different fermentation trials at different stages of fermentation. The bars correspond to the estimated average levels of pH (A), and moisture content (B). The error bars indicate standard errors of the mean. Different letters indicate significant difference ($P < 0.05$) of the pH value or moisture.

was observed in other studies as well (Chen et al., 2010; Knox et al., 2004). In addition, the concentration of lactate was higher when *S. fibuligera* was not present in the fermentation. The accumulation of lactate suggests that *S. fibuligera* stimulates the conversion of lactate to other compounds, such as ethyl lactate. This compound might be abundant in fermentation M. Moreover, *S. fibuligera* could secrete proteinases with an optimum pH 4 (Ismaya et al., 2012), and these enzymes could degrade proteins to peptides and amino acids. As shown in Table S1and Fig. 4J, the content of amino acids such as glycine, histamine, alanine, proline and threonine in fermentation 2 are clearly lower compared to that in fermentation M. This might be associated with the proteinase activity of *S. fibuligera*, especially its high acid proteinase activity (Chi et al., 2009). Several studies have reported that *S. fibuligera* could produce acid protease with an optimum pH in the acidic range (pH 2–5) (Wang et al., 2011a; Yu et al., 2010), it might play an important role in the alcoholic fermentation industry, because it could hydrolyze protein in the fermented mash to liberate amino acids or peptides under the acidic condition (Chi et al., 2009). The production of amino acids not only could provide nitrogen source for the growth of other yeasts, also could react with reducing sugars via Maillard reactions producing flavour compounds, and they might contribute to the soft, delicate and full body of *fen-jiu* as described by Xiong. (2005).

4.2.3. *Wickerhamomyces anomalus and Pichia kudriavzevii*

W. anomalus is regarded as an important aroma producer that is present in a wide range of fermented products (Nyanga et al., 2013; Soka and Irene, 2013). The species is a well-known, significant producer of acetate esters, especially ethyl acetate (Rojas et al., 2001). Ethyl acetate has a fruity, sweet aroma that can contribute to a product's olfactory complexity; thus, enhancing the bouquet of *baijiu* or vinegar. Non-polar compounds were not included in our sample extraction, and therefore, esters were hardly detected by the NMR analysis of our samples. However, we observed that the acetate (flavour precursor) content was higher in fermentation 3 (without *W. anomalus*) than in fermentation M (with *W. anomalus*)

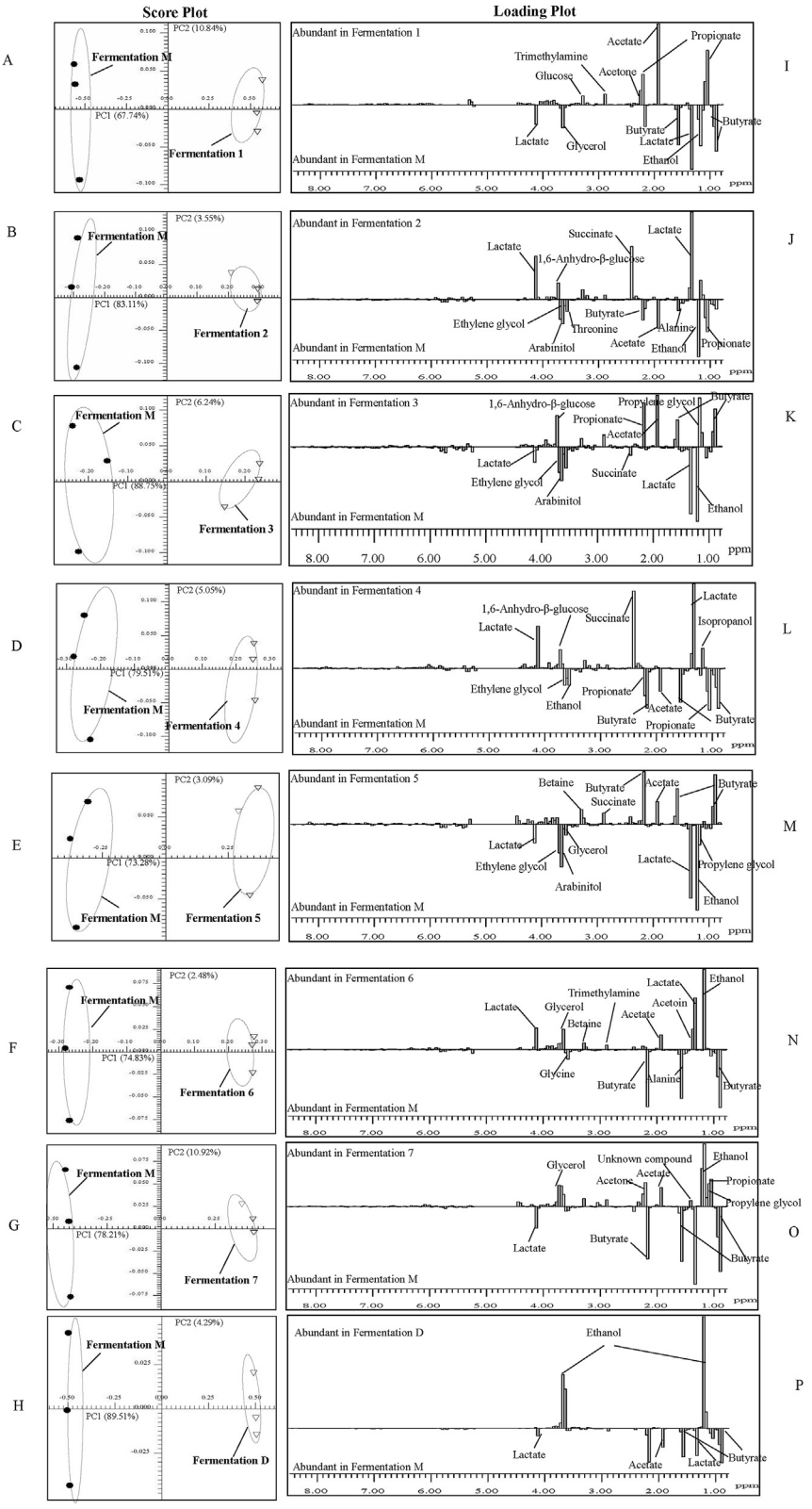

Fig. 4. PCA scores (A–H) and loading (I–O) plots derived from the ^{1}H NMR spectra demonstrating significant statistical changes of metabolites in different fermentation samples at 28 d.

(Fig. 4K). The acetate in fermentation M could be converted by *W. anomalus* to other substances, especially ethyl acetate, which is in agreement with observation made by Passoth et al. (2006). They reported that ethyl acetate could be synthesized by *W. anomalus* from acetate via an esterase. Moreover, Sun (2014) reported that *W. anomalus* could produce both acetate and ethyl acetate in high concentrations in sorghum juice. Ethanol and ethyl acetate may be responsible for the antimicrobial activity of *W. anomalus* (Coda et al., 2011), as the species is a highly competitive yeast able to inhibit a variety of other microorganisms, such as *Penicillium* and *Aspergillus* species (Passoth et al., 2011). This may explain why *Penicillium* and *Aspergillus* species were present at low abundance in both *Daqu* and fermented grains. If so, *W. anomalus* also contributes to the safety of the *Daqu* products, as *Penicillium* and *Aspergillus* are associated with mycotoxin production (Moss, 2002).

Pichia kudriavzevii is another good ethanol producer (Yuangsaard et al., 2013), which is confirmed by our results (Fig. 4L) that showing a higher ethanol concentration in fermentation M if compared to fermentation 4 (without *P. kudriavzevii*).

4.2.4. *Bacillus licheniformis*

B. licheniformis was reported to be widely present in different types of *Daqu*, zaopei, and other fermented grains (Wang et al., 2011b). We observed that this species has a high starch degrading ability, particularly due to high α-amylase activities (i.e., greater than 0.5 U/g, Fig. 1). Starch comprises 65–81% of the total weight of sorghum grains (Cao et al., 2010). The amylases produced by *B. licheniformis* were reported to yield maltose, maltotriose, and maltodextrins (Coda et al., 2011) from starch. Although maltose is expected to be fermented by other microorganisms at the end of the fermentation, the addition of *B. licheniformis* to the fermentation starter caused maltose to accumulate as the main by-product of the initial phase of the fermentation process (see Table S1). During fermentation, *B. licheniformis* potentially produces acetate and lactate (Shen, 2003; Yan et al., 2013), which may explain why the lactate concentration increased in the fermentation trial with *B. licheniformis* (i.e. fermentation M). Lactate is an important substrate for esterification into the main aroma compound ethyl lactate found in light-flavour *baijiu* such as *fen-jiu* (Yan et al., 2013). Ethyl lactate is mainly produced via esterifying enzymes secreted by other microorganisms, such as butyric acid bacteria, during the fermentation of *baijiu* (Sun, 2014). Therefore, *B. licheniformis* most likely makes an important contribution to the formation of flavours and aromas of *baijiu* and vinegar. Ethanol production increased when *B. licheniformis* was added, which could be the result of synergistic effects between *S. cerevisiae* and *B. licheniformis* (Ling, 2013). *B. licheniformis* could promote the production of ethanol and acids (acetic acid, caproic acid, octanoic acid and azelaic acid) by *S. cerevisiae* (Ling, 2013), which may explain the lowest ethanol concentration (0.7 mmol/g) found in fermentation 5 (the one without *B. licheniformis*).

4.2.5. *Lactobacillus plantarum and Pediococcus pentosaceus*

The two LAB species *Lactobacillus plantarum* and *P. pentosaceus* are frequently encountered in *Daqu* and fermented grains (Li et al., 2013; Zheng et al., 2012, 2014). Amylolytic activity of *L. plantarum* strains was reported (Li et al., 2014b) and this species can be regarded as a producer of amylolytic enzymes in the fermentation of sorghum, as was also demonstrated in our screening (see Fig. 1). Our results also showed its proteolytic activity during fermentation, as amino acids, such as glycine and alanine, were produced (Fig. 4N). Alanine is used as a sweetener in the food industry and was frequently found in *Daqu* and its derived products (Mukherjee et al., 2009); therefore, LAB might contribute little to the final flavour of *baijiu*, especially light-flavour *baijiu*, such as *fen-jiu*, as this type of *baijiu* has a pure and sweet taste (Xiong, 2005). The PCA analysis of metabolite profiles of fermentations M and 6 revealed one unexpected result: the concentration of lactate was higher when *L. plantarum* was absent. One possible reason for this is that *L. plantarum* could convert lactate to acetate under limited oxygen conditions (Goffin et al., 2004; Liu, 2003).

P.pentosaceus is found in many cereal-based fermented foods, such as *uji* and *ben-saalga* (Nout, 2009); mainly contributing to the acidification of the raw materials. The pH of fermentation 7 reached approximately pH 6, whereas the pH of the other fermentation trials except fermentation 1 was approximately 4.3 (Fig. 3). This indicates that *P. pentosaceus* can lead to a pH decrease during alcoholic fermentation, similar to that caused by *S. cerevisiae*, and this is mainly due to the production of lactic acid. In contrast to *L. plantarum*, *P. pentosaceus* seems incapable to convert lactate; instead, lactate accumulates as an end product.

The metabolite profile of fermentation D (with *Daqu*) was also compared with that of fermentation M (Fig. 4H). The results indicated a higher ability of *Daqu* to produce ethanol when compared to that formed by the blend of all seven species (*S. cerevisiae*, *S. fibuligera*, *W. anomalus*, *P. kudriavzevii*, *B. licheniformis*, *P. pentosaceus*, and *L. plantarum*) (Fig. 4P). *Daqu* also contains moulds, that are associated with strong amylase activity, such as *Lichtheimia corymbifera* (Zheng et al., 2012). As a result, more fermentable sugars may be generated for use in the alcoholic fermentation by *S. cerevisiae* resulting in higher ethanol yields. However, fermentation M tends to produce more acid, especially lactate and acetate, instead of ethanol (Fig. 4P). The reason for this observation could be the high proportion of *L. plantarum* and *P. pentosaceus* added to the mixtures. As described previously, about 10^7 CFU/g *l. plantarum* and *P. pentosaceus* were added, which is ten times higher than in *Daqu* (10^6 CFU/g). LAB have a very strong ability to compete with *S. cerevisiae* to use glucose as a carbon source for their growth. As a consequence, more lactate and acetate could accumulate and less glucose could be used for the production of ethanol.

The outcomes of fermentations 1–7 were significantly different from each other (Fig. S1), indicating that each species plays a particular role during mixed fermentations. Based on the statistical analysis, adding or removing any of the species resulted in significantly different metabolite profiles. The selected *S. cerevisiae* 1, *S. fibuligera* 4, *W. anomalus* 29, *P. kudriavzevii* 12, *B. licheniformis* 128, *P. pentosaceus* 6, and *L. plantarum* 1 strains may be regarded as important for *Daqu*. However, the behavior of *Daqu* could not yet be simulated completely. Further study involving the additional effect of filamentous fungi will be required.

Propositions

1. Of all the *Daqu* types, *Fen-Daqu* is the best model to investigate the microbial diversity in *Daqu* (this thesis).
2. *Daqu* can't be replaced by simply mixing functional microorganisms (this thesis).
3. Criticism can do more harm than good to children.
4. Modern electronic devices such as the mobile phone and tablet computer reduce communications among families and friends.
5. Traditional fermented food have a long history of safe use, doesn't guarantee safety.
6. Although next generation sequencing (NGS) could provide large quantities of microbial data, its use needs to be carefully consideration.
7. Good microorganisms can become to be bad one when the environment changed.
8. CCA is a powerful approach to analyse the relationship between microbial composition and environmental conditions.

Acknowledgements

We thank Wu Xiao-He for technical assistance and Shanxi Xinghuacun *Fen-jiu* Group Company for *Daqu* sampling assistance and advice. This project was supported by the National Natural Science Foundation of China (31371716 and 31671829) and Science-Technology Foundation of Beijing Municipal Commission of Education (KM201710858002).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.03.015.

References

Acourene, S., Ammouche, A., 2012. Optimization of ethanol, citric acid, and α-amylase production from date wastes by strains of *Saccharomyces cerevisiae, Aspergillus niger*, and *Candida guilliermondii*. J. Ind. Microbiol. Biotechnol. 39, 759–766.

Cao, Y.G., Hu, Y.G., Ma, Y.H., Du, X.W., Ma, E.B., Quan, Z.X., Zhang, S.W., 2010. Variation of aromatic components in solid phase fermented grains during fermentation of *Fen* liquor. Food Sci. 31, 367–371 (in Chinese).

Chen, L., Chi, Z.M., Chi, Z., Li, M., 2010. Amylase production by *Saccharomycopsis fibuligera* A11 in solid-state fermentation for hydrolysis of cassava starch. Appl. Biochem. Biotechnol. 162, 252–263.

Chi, Z., Chi, Z., Liu, G., Wang, F., Ju, L., Zhang, T., 2009. *Saccharomycopsis fibuligera* and its applications in biotechnology. Biotechnol. Adv. 27, 423–431.

Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., Gobbetti, M., 2011. Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: identification of novel compounds and long-term effect during storage of wheat bread. Appl. Environ. Microbiol. 77, 3484–3492.

Gül, H., Özçelik, S., Sağdıç, O., Certel, M., 2005. Sourdough bread production with lactobacilli and *S. cerevisiae* isolated from sourdoughs. Process Biochem. 40, 691–697.

Goffin, P., Lorquet, F., Kleerebezem, M., Hols, P., 2004. Major role of NAD-dependent lactate dehydrogenases in aerobic lactate utilization in *Lactobacillus plantarum* during early stationary phase. J. Bacteriol. 186, 6661–6666.

Huffer, S., Clark, M.E., Ning, J.C., Blanch, H.W., Clark, D.S., 2011. Role of alcohols in growth, lipid composition, and membrane fluidity of yeasts, bacteria, and archaea. Appl. Environ. Microbiol. 77, 6400–6408.

Ismaya, W.T., Hasan, K., Subroto, T., Natalia, D., Soemitro, S., 2012. Chromatography as the major tool in the identification and the structure function relationship study of amylolytic enzymes from *Saccharomycopsis fibuligera* R64. INtech 272–293.

Jančič, S., Zalar, P., Kocev, D., Schroers, H.J., Džeroski, S., Gunde-Cimerman, N., 2015. Halophily reloaded: new insights into the extremophilic life-style of *Wallemia* with the description of *Wallemia hederae* sp. nov. Fungal Divers. 76, 97–118.

Kim, H.S., Kim, N.R., Choi, W., 2011. Total fatty acid content of the plasma membrane of *Saccharomyces cerevisiae*; is more responsible for ethanol tolerance than the degree of unsaturation. Biotechnol. Lett. 33, 509–515.

Knox, A.M., du Preez, J.C., Kilian, S.G., 2004. Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. Enzym. Microb. Technol. 34, 453–460.

Le, V.D., Zheng, X.W., Ma, K., Chen, J.Y., Han, B.Z., Nout, M.J.R., 2011. Characterization of *Fen-Daqu* through multivariate statistical analysis of ^{1}H NMR spectroscopic data. J. Inst. Brew. 117, 516–522.

Li, P., Li, S., Cheng, L., Luo, L., 2014a. Analyzing the relation between the microbial diversity of *Daqu* and the turbidity spoilage of traditional Chinese vinegar. Appl. Microbiol. Biotechnol. 98, 6073–6084.

Li, X.R., Ma, E.B., Yan, L.Z., Meng, H., Du, X.W., Quan, Z.X., 2013. Bacterial and fungal diversity in the starter production process of Fen liquor, a traditional Chinese liquor. J. Microbiol. 51, 430–438.

Li, X.R., Ma, E.B., Yan, L.Z., Meng, H., Du, X.W., Zhang, S.W., Quan, Z.X., 2011. Bacterial and fungal diversity in the traditional Chinese liquorfermentation process. Int. J. Food Microbiol. 146, 31–37.

Li, Z., Bai, Z., Wang, D., Zhang, W., Zhang, M., Lin, F., Gao, L., Hui, B., Zhang, H., 2014b. Cultivable bacterial diversity and amylase production in three typical *Daqus* of Chinese spirits. Int. J. Food Sci. Technol. 49, 776–786.

Ling, J., 2013. Interactions between *Bacillus Licheniformis* and *Saccharomyces cerevisiae* and Their Application in Liqour Fermentation. Jiang Nan University, Jiangnan.

Liu, S., Qureshi, N., 2009. How microbes tolerate ethanol and butanol. N. Biotech. 26, 117–121.

Liu, S.Q., 2003. Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. Int. J. Food Microbiol. 83, 115–131.

Liu, X., Guo, K.L., Zhang, H.X., 2012. Determination of microbial diversity in *Daqu*, a fermentation starter culture of Maotai liqour, using nested PCR-denaturing gradient gel electrophoresis. World J. Microbiol. Biotechnol. 28, 2375–2381.

Moss, M.O., 2002. Mycotoxin review – 1. *Aspergillus* and *Penicillium*. Mycologist 16, 116–119.

Mukherjee, A.K., Borah, M., Rai, S.K., 2009. To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. Biochem. Eng. J. 43, 149–156.

Nie, Z., Zheng, Y., Wang, M., Han, Y., Wang, Y., Luo, J., Niu, D., 2013. Exploring microbial succession and diversity during solid-state fermentation of Tianjin duliu mature vinegar. Bioresour. Technol. 148, 325–333.

Nout, M.J.R., 2009. Rich nutrition from the poorest - cereal fermentations in africa and asia. Food Microbiol. 26, 685–692.

Nyanga, L.K., Nout, M.J.R., Smid, E.J., Boekhout, T., Zwietering, M.H., 2013. Fermentation characteristics of yeasts isolated from traditionally fermented *masau* (*Ziziphus mauritiana*) fruits. Int. J. Food Microbiol. 166, 426–432.

Passoth, V., Fredlund, E., Druvefors, U.Ä., Schnürer, J., 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 6, 3–13.

Passoth, V., Olstorpe, M., Schnürer, J., 2011. Past, present and future research directions with *Pichia anomala*. Antonie Leeuwenhoek 99, 121–125.

Rojas, V., Gil, J.V., Piñaga, F., Manzanares, P., 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int. J. Food Microbiol. 70, 283–289.

Saelim, K., Dissara, Y., H-Kittikun, A., 2008. Saccharification of cassava starch by *Saccharomycopsis fibuligera* YCY1 isolated from Loog-Pang (rice cake starter). Songklanakarin J. Sci. Technol. 30, 65–71.

Shen, Y.F., 2003. Study on the Formation Mechanism of Four Main Kinds of Ethyl Esters in the Fermentation of Liquors. Liquor-making Science and Technology, vol. 119 (in Chinese).

Smid, E.J., Kleerebezem, M., 2014. Production of aroma compounds in lactic fermentations. Annual Review of Food Science and Technology 5, 313–326.

Soka, S., Irene, M., 2013. Molecular analysis of yeasts from Indonesian cassava and glutinous rice tapé. Food Sci. Technol. 22, 993–997.

Sun, Y.T., 2014. The Fermentation Characteristics of Yeast Species Isolated from *Fen-daqu* during Alcoholic Fermentation. China Agricultural University, Beijing.

Tabassum, R., Khaliq, S., Rajoka, M.I., Agblevor, F., 2014. Solid state fermentation of a raw starch digesting alkaline alpha-amylase from *Bacillus licheniformis* RT7PE1 and its characteristics. Biotechnology Research International.

Torres, S., Baigorí, M.D., Castro, G.R., 2005. Effect of hydroxylic solvents on cell growth, sporulation, and esterase production of *Bacillus licheniformis* S-86. Process Biochem. 40, 2333–2338.

Wang, D.S., Chi, Z., Zhao, S.F., Chi, Z.M., 2011a. Disruption of the acid protease gene in *Saccharomycopsis fibuligera* A11 enhances amylolytic activity and stability as well as trehalose accumulation. Enzym. Microb. Technol. 49, 88–93.

Wang, H.Y., Gao, Y.B., Fan, Q.W., Xu, Y., 2011b. Characterization and comparison of microbial community of different typical Chinese liquor *Daqus* by PCR-DGGE. Lett. Appl. Microbiol. 53, 134–140.

Wu, X.H., Zheng, X.W., Han, B.Z., Vervoort, J., Nout, M.J.R., 2009. Characterization of Chinese liquor starter, "*Daqu*" by flavour type with ^{1}H NMR-based nontargeted analysis. J. Agric. Food Chem. 57, 11354–11359.

Xiong, Z.S., 2005. Research on three flavour type liquor in China (3) Fen flavour liquor- Xinghuacun. Liquor-making Sci. Technol. 7, 17–21 (in Chinese).

Yan, Z., Zheng, X.W., Chen, J.Y., Han, J.S., Han, B.Z., 2013. Effect of different *Bacillus* strains on the profile of organic acids in a liquid culture of *Daqu*. J. Inst. Brew. 119, 78–83.

Yu, X.J., Li, H.J., Li, J., Chi, Z.M., 2010. Overexpression of acid protease of *Saccharomycopsis fibuligera* in Yarrowia lipolytica and characterization of the recombinant acid protease for skimmed milk clotting. Biotechnol. Bioproc. Eng. 15, 467–475.

Yuangsaard, N., Yongmanitchai, W., Yamada, M., Limtong, S., 2013. Selection and characterization of a newly isolated thermotolerant *Pichia kudriavzevii* strain for ethanol production at high temperature from cassava starch hydrolysate. Antonie Leeuwenhoek 103, 577–588.

Zheng, X.W., Tabrizi, M.R., Nout, M.J.R., Han, B.Z., 2011. *Daqu* - a traditional Chinese liquor fermentation starter. J. Inst. Brew. 117, 82–90.

Zheng, X.W., Yan, Z., Han, B.Z., Zwietering, M.H., Samson, R.A., Boekhout, T., Nout, M.J.R., 2012. Complex microbiota of a Chinese "Fen" liquor fermentation starter (*Fen-Daqu*), revealed by culture-dependent and culture-independent methods. Food Microbiol. 31, 293–300.

Zheng, X.W., Yan, Z., Nout, M.J.R., Smid, E.J., Zwietering, M.H., Boekhout, T., Han, J.S., Han, B.Z., 2014. Microbiota dynamics related to environmental conditions during the fermentative production of *Fen-Daqu*, a Chinese industrial fermentation starter. Int. J. Food Microbiol. 182–183, 57–62.

Zheng, X.W., Han, B.Z., 2016. *Baijiu* (白酒), Chinese liquor: history, classification and manufacture. Journal of Ethnic Food 19–25.