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Monitoring the Ecology of *Bacillus* During *Daqu* Incubation, a Fermentation Starter, Using Culture-Dependent and Culture-Independent Methods

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***Daqu*, a traditional fermentation starter, has been used to produce attractively flavored foods such as vinegar and Chinese liquor for thousands of years. Although *Bacillus* spp. are one of the dominant microorganisms in *Daqu*, more precise information is needed to reveal why and how *Bacillus* became dominant in *Daqu*, and next, to assess the impact of *Bacillus* sp. on *Daqu* and its derived products. We combined culture-dependent and culture-independent methods to study the ecology of *Bacillus* during *Daqu* incubation. Throughout the incubation, 67 presumptive *Bacillus* spp. isolates were obtained, 52 of which were confirmed by 16S rDNA sequencing. The identified organisms belonged to 8 *Bacillus* species: *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. megaterium*, *B. pumilus*, and *B. anthracis*. A primer set specific for *Bacillus* and related genera was used in a selective PCR study, followed by a nested DGGE PCR targeting the V9 region of the 16S rDNA. Species identified from the PCR-DGGE fingerprints were related to *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus, B. benzoevorans*, and *B. foraminis*. The predominant species was found to be *B. licheniformis*. Certain *B. licheniformis* strains exhibited potent antimicrobial activities. The greatest species diversity occurred at the *Liangmei* stage of *Daqu* incubation. To date, we lack sufficient knowledge of *Bacillus* distribution in *Daqu*. Elucidating the ecology of *Bacillus* during *Daqu* incubation would enable the impact of *Bacillus* on *Daqu* to be accessed, and the quality and stabilization of *Daqu*-derived products to be optimized.**

Key words: Microbial ecology, *Bacillus*, fermentation starter, *Daqu*, PCR-DGGE

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Qu is a traditional fermentation starter, commonly used in Asia to ferment a variety of cereal substrates. The fermentation process produces foods with attractive flavors, such as vinegar, Chinese liquor, soy sauce, and rice wine. *Daqu*, a type of grain *Qu*, has been used for thousands of years [38]. *Daqu* is typically made from raw barley and pea, incubated under uncontrolled conditions without adding defined starter microorganisms. *Daqu* incubation is commonly divided into seven stages: *Woqu* (WQ), *Shangmei* (SM), *Liangmei* (LM), *Chaohuo* (CH), *Dahuo* (DH), *Houhuo* (HH), and *Yangqu* (YQ). The entire incubation period is about one month [22]. Following the incubation, *Daqu* is further fermented by mixing with cooked cereals at a proportion of approximately 9–10%. Previous culture-independent and culture-dependent studies have revealed the presence of yeasts, molds, bacteria, and actinomycetes in *Daqu*. Of these, the bacteria, especially thermophilic *Bacillus*, are present in higher numbers than fungi [22, 25, 37]. Although *Bacillus* spp. are among the dominant microorganisms in *Daqu* (up to 7–8 log CFU/g) [37, 38], more precise information is needed to reveal why and how this situation evolved. Armed with this knowledge, the impact of *Bacillus* on *Daqu* and its derived products can be evaluated.

Bacillus spp. are ubiquitous spore-forming bacteria associated with a variety of food products [1, 14, 21]. Usually, they are implicated in spoilage of food products and cases of food poisoning [3]. They are also used to produce commercial enzymes, such as amylases, proteases, amd lipases. Indeed, enzyme production by *Bacillus* may play a role in traditional food fermentation [11, 17, 18].

In this study, we combined culture-dependent and culture-independent methods to investigate the ecology of *Bacillus* throughout *Daqu* incubation. To more precisely identify the organisms involved, a nested-PCR was used, targeted specifically at the 16S rDNA genes of *Bacillus* and related genera. The antimicrobial spectra of certain

Bacillus strains were also tested in order to confirm the nature of their dominance distribution. The results of this study may assist in improving the quality and stabilization of *Daqu*-derived products.

Materials and Methods

Sampling

The *Daqu* samples used in this study were collected in October 2009 from the Shanxi Xinghuacun Fenjiu Distillery Co. Ltd. (Fenyang, Shanxi, China). Each *Daqu* block is approximately 27.5 × 16 × 5.5 cm^3 and weighs about 1.7 kg. *Daqu* was produced and matured in stacked layers by traditional methods. Samples were collected at the end of the WQ, SM, LM, CH, DH, HH, and YQ stages. The temperatures of the samples were recorded by a mini infrared thermometer gun (UNI-T UT301A, Beijing) at the time of sampling. Samples were randomly selected from each upper, middle, and lower stacked layer, and ground and pooled to provide an experimental *Daqu* powder sample. For culture-based investigations, the samples were stored at 4°C and analyzed within 48 h. For culture-independent investigations, the samples were stored at −20°C prior to further analyses.

Enumeration and Isolation

Culture-based microbiological investigations were conducted at all seven stages of *Daqu* incubation. Ten grams of powdered sample was added to 90 ml of sterile PPS (peptone physiological salt) solution containing 1 g/l peptone (Oxoid LP0034; Oxoid, Basingstoke, Hampshire, UK) and 8.5 g/l NaCl in a Stomacher Lab-blender 400 and homogenized. Appropriate serial dilutions were prepared from the same diluent. Unless otherwise indicated, 1 ml aliquots of the diluted suspensions were mixed with molten (45°C) medium and poured onto duplicate counting plates of the media described below.

Total aerobic and anaerobic bacteria were enumerated on plate count agar (PCA; Oxoid CM0035). Plates were incubated at 30°C for 1−2 days.

For the enumeration of bacterial spores, the first decimal dilution was pasteurized at 80°C for 10 min [5], and serially diluted. Then 100 µl of the appropriate dilution was pour-plated onto PCA (Oxoid CM0035) topped with an additional layer of 1.5% bacteriological agar (Oxoid LP0011). All plates were prepared in duplicate and incubated at 37°C for 2−4 days.

Fungi (yeasts and molds) were enumerated on Rose Bengal chloramphenicol agar (RBCA; Oxoid CM0549), to which 100 mg/l chloramphenicol (Oxoid SR0078E) was added [37]. Then 100 µl of diluted suspensions were spread-plated onto this medium and incubated at 25°C for 2−4 days.

Duplicate counts were made for each of the duplicated samples, yielding four counts per sample. The results are reported as the means ± SD (n = 4). For plates of bacterial spores containing around 100 colonies, the number of randomly selected isolates was the square root of the total number of colonies. Isolates were grown in nutrient broth (NB, Oxoid CM0001B) media under the conditions applied previously for plate counting.

Identification of the Isolates by 16S rDNA Sequence Analyses

Presumptive *Bacillus* spp. isolates were grown in nutrient broth medium supplemented with $MnCl_2$ (50 mg/l) to encourage sporulation. DNA was extracted from endospore-forming isolates using the Bacteria Genomic DNA Purification Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. An approximately 1500 bp fragment of the 16S rDNA was amplified using forward primer B-for and reverse primer B-rev. The primers used in this study are shown in Table 1. The sequencing of the 16S rDNA fragment was performed by Beijing Center for Physical and Chemical Analysis (Beijing, China). Sequence similarity was determined by accessing the BLAST database from GenBank.

Identification by Culture-Independent Methods

Total DNA extraction. For culture-independent investigations, 2 g of each *Daqu* sample was suspended in 30 ml of PBS, and total DNA was extracted by a minor modification of the method of Wang *et al.* [29]. The collected DNA was air-dried and dissolved in 30 µl of sterile water. DNA concentration was assayed by ultraviolet-visible spectrophotometers (Unico, Shanghai, China) and diluted to an approximate final concentration of 10 ng/µl.

PCR. The first PCR experiment adopted primers pB/pH (Table 1), designed specifically for *Bacillus* and related genera (such as *Alicyclobacillus*, *Brevibacillus*, and *Clostridium*). The first PCR (referred to as "selective PCR") was performed in a 50 µl reaction mixture containing 37.75 µl of ddH_2O, 5 µl of PCR buffer, 4 µl of dNTP (2 mmol/l), 1 µl of each primer (10 µmol/l), 1 µl of DNA template, and 0.25 µl of Ex Taq DNA polymerase (5 U/µl) (Takara, Japan). PCR reactions were undertaken in a AG 223B1 Thermoblock (Eppendorf, NY, USA) with PCR conditions as follows: initial denaturation of double-stranded DNA for 5 min at 94°C; 35 cycles,

Table 1. PCR primers used in this study.

Primer	Sequence (5' → 3')	Aims	Reference
B-for (F)	AACGCGAAGAACCTTAC	Amplify 16S rDNA and sequencing for bacteria	[16]
B-rev (R)	CGGTGTGTACAAGACCC		
pB (F)	CGATGCGTAGCCGACCTGAG	Amplify 16S rDNA and sequencing especially for *Bacillus* and related genera	[2]
pH (R)	AAGGAGGTGATCCAGCCGCA		
Ec1055 (F)	ATGGCTGTCGTCAGCT	DGGE PCR for *Bacillus* and related genera	[4]
Ec1392 (R)	CGCCCGCCGCGCCCCGCGCCCGTCCCGC CGCCCCCGCCCGACGGGCGGTGTGTAC		

F, forward primer; R, reversed primer. The GC clamp is underlined.

each consisting of 30 sec at 94°C, 45 sec at 56°C, and 90 sec at 72°C; and extension of incomplete products for 7 min at 72°C, followed by cooling at 4°C.

The product of this selective PCR was diluted to 1:100 and used as template for a second PCR (referred to as "DGGE PCR"), using primers Ec1055 and Ec1392 (Table 1). PCR was performed in a 50 µl reaction mixture containing 37.75 µl of ddH_2O, 5 µl of PCR buffer, 4 µl of dNTP (2 mmol/l), 1 µl of each primer (10 µmol/l), 1 µl of DNA template, and 0.25 µl of Ex Taq DNA polymerase (5 U/µl) (Takara, Japan). PCR reactions were undertaken in a AG 223B1 Thermoblock (Eppendorf) with PCR conditions as follows: initial denaturation of double-stranded DNA for 5 min at 94°C; 35 cycles, each consisting of 30 sec at 94°C, 45 sec at 56°C, and 1 min at 72°C; and extension of incomplete products for 7 min at 72°C followed by cooling at 4°C.

DGGE analyses. DGGE was performed using the Dcode system (Bio-Rad, Hercules, CA, USA) on 20 cm × 16 cm × 1 mm gels. Electrophoresis was performed at 60°C in 0.5× TAE buffer (20 mmol/l Tris-acetate, 2 mmol/l EDTA; pH 8.0) using 8% polyacrylamide gels containing a 30–60% urea-formamide linear denaturing gradient [where 100% corresponds to 7 mol/l urea and 40% (v/v) formamide]. Samples were run for 16 h at 85 V.

Following electrophoresis, the gels were stained with $AgNO_3$ solution as follows: The gel was fixed and shaken gently in 1× Cairn's fixation solution (200 ml 96% ethanol; 10 ml acetic acid; 40 ml demi-water) for 3 min. The gel was transferred to a freshly made $AgNO_3$ staining solution (2 g $AgNO_3$ per liter), shaken gently for 10 min, and briefly rinsed in water. The stained gel was developed in a freshly made developing solution (10 mg $NaBH_4$, 250 ml 1.5% NaOH solution, 750 µl formaldehyde) until the desired exposure was achieved.

Bands of interest were excised from the gel and boiled for 30 min, and then incubated overnight in 30 µl 0.25× TAE buffer (10 mmol/l Tris-acetate, 1 mmol/l EDTA; pH 8.0) at 4°C to allow diffusion of the DNA.

Sequencing of DGGE bands. Eluted DNA was re-amplified using the corresponding DGGE primers (Ec1055 and Ec1392), without the GC clamp. The PCR products were sequenced at the Beijing Center for Physical and Chemical Analysis (Beijing, China). Sequence identity was determined by accessing the nucleotide BLAST database of GenBank. To confirm the reproducibility of the DGGE fingerprints, the DNA isolated from the different DGGE bands was PCR-amplified three times.

Determination of Antimicrobial Activity

To examine the antimicrobial activity, 9 native *B. licheniformis* strains (YP-P-01, YP-P-02, YP-P-03, YP-P-04, YP-P-05, YP-P-07, YP-P-09, YP-P-11, YP-P-12) isolated at the end of *Daqu* incubation were used. *B. licheniformis* strains were grown in 100 ml of NB (Oxoid CM0001B) at 37°C, 120 rpm for 24 h, respectively. Next, the cell-free supernatant was collected by centrifugation at 10,000 rpm for 15 min at 4°C.

The antimicrobial activities were investigated against 7 indicators of fungi and 11 indicators of bacteria (Table 4). All microorganisms were native microorganisms previously isolated from naturally fermented *Daqu* (Shanxi Xinghuacun Fenjiu Distillery Co. Ltd., Fenyang, Shanxi, China) and identified as described by Zheng *et al*. [37].

The agar diffusion assay described by Guo *et al*. [6] was used to test the antimicrobial activities of the cell-free supernatants. The indicator strains were grown at the corresponding conditions (Table 4) for 2–4 days. Sterile Oxford cups were placed on these agar plates and cell-free supernatant (100 µl) of each *B. licheniformis* strain was added into each Oxford cup. After incubation for 12 h at the optimum growth temperature for the test organism, the presence of a clear zone around the cup (including that of the "Oxford cup" 7.8 mm) was measured and the results were reported in mm. The experiment was performed in duplicate on three separate occasions.

RESULTS

Enumeration of Representative Bacteria and Fungi on Selective Media

The culture-dependent methods, namely enumeration of bacteria, bacterial spores, and fungi, revealed gradual changes of within the microorganism community throughout *Daqu* incubation. Fig. 1 presents the dynamics of viable counts during the incubation, together with the temperatures of each sample at the respective stages of incubation. The total viable numbers of aerobic and anaerobic bacteria were significantly increased during all stages except CH, where they were reduced. Initial counts of bacterial endospores were around 4 log CFU/g (at the WQ stage), increasing rapidly to 9 log CFU/g at the HH stage, and remaining constant thereafter. The total fungal counts increased rapidly from the initial WQ stage to SM, and then decreased (from LM to YQ). During the first three stages (WQ, SM, and LM), fungi presented in higher numbers than bacterial spores. After the CH stage, the bacterial spore counts exceeded those of fungi. Also from Fig. 1, we observe that the sample temperature increased from a mere 20°C at WQ to its maximal value of 55°C at HH. Following the HH stage, the temperature hovered around 30°C, apart from a curiously anomalous slight dip at the LM stage.

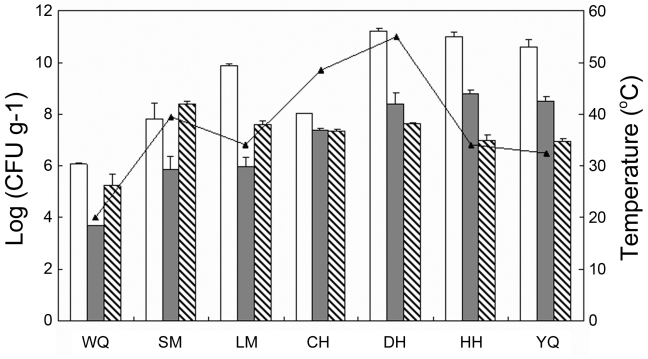

Fig. 1. Microbial community composition and temperatures at each stage of *Daqu* incubation.
WQ: *Woqu*; SM: *Shangmei*; LM: *Liangmei*; CH: *Chaohuo*; DH: *Dahuo*; HH: *Houhuo*; YQ: *Yangqu*. ▭: Total Viable Count; ▬: Sport-forming Bacteria; ▨: Fungi; ▲: Temperature.

Table 2. Species-level identification of 52 isolates (n = 67) based on partial 16S rDNA sequencing of the *Daqu* samples.

Step	Sample code	Sequencing	Accession no.	% Identity
WQ (n = 8; k = 5)	WQ-P-02	*Bacillus licheniformis*	AB553280.1	99
	WQ-P-03	*Bacillus licheniformis*	EU379276.1	99
	WQ-P-05	*Bacillus licheniformis*	HM006908.1	99
	WQ-P-06	*Bacillus licheniformis*	GU967452.1	100
	WQ-P-07	*Bacillus pumilus*	GU125637.1	99
SM (n = 8; k = 7)	SM-P-01	*Bacillus licheniformis*	AY871102.1	99
	SM-P-02	*Bacillus pumilus*	EU379276.1	100
	SM-P-03	*Bacillus pumilus*	GU290547.1	99
	SM-P-04	*Bacillus licheniformis*	GU967452.1	99
	SM-P-05	*Bacillus licheniformis*	GQ222400.1	99
	SM-P-06	*Bacillus licheniformis*	AY871102.1	99
	SM-P-07	*Bacillus subtilis*	EF032678.1	99
LM (n = 11; k = 9)	LM-P-01	*Bacillus pumilus*	GU084168.1	99
	LM-P-02	*Bacillus licheniformis*	GU967452.1	100
	LM-P-03	*Bacillus licheniformis*	GU967452.1	99
	LM-P-04	*Bacillus pumilus*	GU084168.1	100
	LM-P-05	*Bacillus cereus*	GQ199590.1	99
	LM-P-06	*Bacillus cereus*	GQ355961.1	99
	LM-P-08	*Bacillus megaterium*	DQ105968.1	99
	LM-P-10	*Bacillus megaterium*	DQ105968.1	99
	LM-P-11	*Bacillus anthracis*	CP001598.1	99
CH (n = 9; k = 6)	CH-P-01	*Bacillus licheniformis*	AY871102.2	99
	CH-P-03	*Bacillus licheniformis*	GU967452.1	99
	CH-P-04	*Bacillus megaterium*	EU931553.1	99
	CH-P-05	*Bacillus licheniformis*	GU967452.1	99
	CH-P-06	*Bacillus circulans*	AY043084.1	99
	CH-P-08	*Bacillus licheniformis*	GU967449.1	100
DH (n = 10; k = 9)	DH-P-01	*Bacillus licheniformis*	FJ435674.1	99
	DH-P-02	*Bacillus licheniformis*	AB374301.1	99
	DH-P-03	*Bacillus licheniformis*	GU967449.1	99
	DH-P-04	*Bacillus licheniformis*	GU967452.1	99
	DH-P-05	*Bacillus licheniformis*	HM055609.1	99
	DH-P-06	*Bacillus licheniformis*	GU967452.1	99
	DH-P-07	*Bacillus licheniformis*	DQ870721.1	100
	DH-P-08	*Bacillus licheniformis*	GU967452.1	100
	DH-P-09	*Bacillus licheniformis*	CP001598.1	99
HH (n = 9; k = 6)	HH-P-02	*Bacillus licheniformis*	HM006868.1	99
	HH-P-03	*Bacillus licheniformis*	GU967449.1	100
	HH-P-04	*Bacillus pumilus*	EU379276.1	100
	HH-P-06	*Bacillus licheniformis*	AB553280.1	99
	HH-P-07	*Bacillus licheniformis*	AF276309.1	99
	HH-P-09	*Bacillus pumilus*	GQ903421.1	98
YQ (n = 12; k = 10)	YQ-P-01	*Bacillus licheniformis*	GU967449.1	99
	YQ-P-02	*Bacillus licheniformis*	AY750906.1	99
	YQ-P-03	*Bacillus licheniformis*	GQ222400.1	99
	YQ-P-04	*Bacillus licheniformis*	GU967449.1	100
	YQ-P-05	*Bacillus licheniformis*	EF472268.1	99
	YQ-P-07	*Bacillus licheniformis*	GU967449.1	100
	YQ-P-08	*Bacillus amyloliquefaciens*	EU257436	99
	YQ-P-09	*Bacillus licheniformis*	GQ222400.1	99
	YQ-P-11	*Bacillus licheniformis*	GU967449.1	99
	YQ-P-12	*Bacillus licheniformis*	AY8971102.1	100

WQ: *Woqu*; SM: *Shangmei*; LM: *Liangmei*; CH: *Chaohuo*; DH: *Dahuo*; HH: *Houhuo*; YQ: *Yangqu*.
n = the total number of isolates; k = the total number of isolates identified as *Bacillus.*

Identification of the Bacterial Isolates

Table 2 lists the identified *Bacillus* species based on partial sequence alignment of 16S rDNA at each stage. Of the 67 isolates, 52 were identified as *Bacillus* by 16S rDNA sequence comparison against the BLAST database entries; the remaining isolates comprised *Staphylococcus* and *Acetobacter* spp. Most of the sequenced isolates showed high similarity (99–100%) to GenBank sequences. The identified *Bacillus* organisms belonged to 8 species: *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. megaterium*, *B. pumilus*, and *B. anthracis*. From Table 2, it is seen that *B. licheniformis* predominated throughout *Daqu* incubation, representing approximately 70% of all isolates. The *Bacillus* community was most diverse at the LM stage with five different species detected, most of which declined to undetectable levels in successive production stages.

DGGE Analyses

The DGGE patterns of the production stages are presented in Fig. 2. Label identifications are provided in Table 3. The results of the two approaches generally coincide. Total DNA extracted from each *Daqu* incubation stage was used in a nest-PCR with primers pB/pH (selective PCR) and Ec1055/Ec1392 (DGGE PCR), to amplify a section of the V9 region of the 16S rDNA for DGGE analyses. A characteristic snapshot of the band patterns is shown in Fig. 2, and the closest GenBank relatives of selected DGGE bands are shown in Table 3. PCR-DGGE analyses of *Daqu* DNA confirmed the presence of the easily cultured species, besides revealing several uncultured *Bacillus* spp.

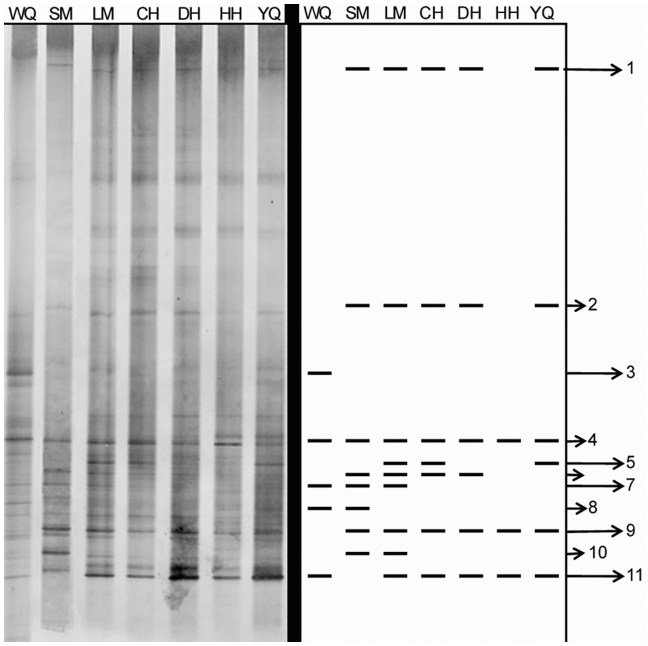

Fig. 2. DGGE profiles (30–60% denaturant) representing 16S rDNA fragments of the *Daqu* samples.
Bands excised from the gel and sequenced (see also Table 3) are numbered.

Eleven bands were resolved in the PCR-DGGE analysis, corresponding to 6 species: *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. benzoevorans*, and *B. foraminis.* Uncultured *Bacillus* and other genera were also detected by DGGE. The diversity of *Bacillus* increased from WQ to LM, reaching a maximum of 9 species at the LM stage, after which the diversity decreased until the end of the incubation. *B. licheniformis* was detected at every stage of the incubation, as evidenced by a very intense DGGE band migrating approximately to the end of the DGGE gels. We infer that *B. licheniformis* is a dominant species throughout the entire incubation period. Other *Bacillus* spp. were detected only at specific stages of the incubation. *B. subtilis* was present at all stages except the WQ stage. *B. amyloliquefaciens* was present at all stages except the SM stage. *B. pumilus* was only found at the LM, CH, and YQ stages, whereas *B. benzoevorans* was absent from the WQ, HH, and YQ stages. *B. foraminis* was only found at the WQ, SM, and LM stages. Band No. 2 in Fig. 2 was strong everywhere except at the WQ and HH stages. Unfortunately, this band could not be resolved.

Antimicrobial Spectra of *B. licheniformis* Strains

Antimicrobial activities of the cell-free supernatants of *B. licheniformis* strains against several bacterial, fungal, and yeast strains were tested (Table 5). Only the cell-free supernatants of YQ-P-02, YQ-P-05, and YQ-P-12 did not inhibit all the indicators. The cell-free supernatants of YQ-P-01, YQ-P-04, YQ-P-07, and YQ-P-11 showed inhibition of several indicators, including *B. cereus* LM-P-05, *B. subtilis* SM-P-07, *S. epidermidis* YQ-P-10, *E. coli* SM-V-02, and *S. enterica* WQ-M-06. The cell-free supernatants of YQ-P-03 and YQ-P-09 only inhibited *S. epidermidis* YQ-P-10, *E. coli* SM-V-02, and *S. enterica* WQ-M-06. All

Table 3. Sequence analyses of PCR-DGGE-generated bands from V9 amplification of the *Daqu* samples.

Band no.	Closet relative in GenBank	Accession no.	% Identity
1	Uncultured bacilli bacterium	JF733120.1	97
2	No sequence		
3	Uncultured *Bacillus* sp.	GU936825.1	100
4	*Bacillus licheniformis*	AY871103.1	99
5	*Bacillus pumilus*	EU379276.1	100
6	*Bacillus benzoevorans*	NR_044828.1	98
7	*Bacillus foraminis*	GQ903409.1	99
8	Uncultured bacterium	FM873507.1	100
9	*Bacillus subtilis*	HQ694497.1	100
10	*Bacillus* sp.	FN397517.1	100
11	*Bacillus amyloliquefaciens*	JN559885.1	100

Table 4. Indicator microorganisms.

Indicators	Growth media[a]	Growth temperature
Yeast		
Pichia kudriavzevii LM-05	Malt extract agar	25°C
Saccharomyces cerevisiae WQ-11	Malt extract agar	25°C
Saccharomycopsis fibuligera SM-03	Malt extract agar	25°C
Wickerhamomyces anomalus WQ-10	Malt extract agar	25°C
Filamentous fungi		
Absidia corymbifera HH-07	Malt extract agar	30°C
Rhizomucor pusillus HH-05	Malt extract agar	30°C
Rhizopus stolonifer SM-02	Malt extract agar	30°C
Gram-positive bacteria		
B. cereus LM-P-05	Nutrient agar	37°C
B. pumilus WQ-P-07	Nutrient agar	37°C
B. subtilis SM-P-07	Nutrient agar	37°C
Lactobacillus plantarum LM-M-02	MRS agar	37°C
Micrococcus luteus LM-M-01	Nutrient agar	30°C
Staphylococcus epidermidis YQ-P-10	Nutrient agar	37°C
Gram-negative bacteria		
Acetobacter tropicalis CH-P-02	Nutrient agar	37°C
Escherichia coli SM-V-02	Nutrient agar	37°C
Pediococcus pentosaceus YQ-M-10	Brain-heart infusion agar	37°C
Pseudomonas aeruginosa SM-M-04	Nutrient agar	37°C
Salmonella enterica WQ-M-06	Brain-heart infusion agar	37°C

[a]Brain-heart infusion (BHI) agar containing BHI broth (Oxoid CM0225) and 1.5% of bacteriological agar (Oxoid LP0011) (pH 7.0); Malt extract agar (MEA, Oxoid CM0059); MRS agar (Oxoid CM0361); Nutrient agar (Oxoid CM0003).

Table 5. Antimicrobial spectra of cell-free supernatants of *B. licheniformis* strains.

Indicators	Antimicrobial activity								
	B. licheniformis strains								
	YQ-P-01	YQ-P-02	YQ-P-03	YQ-P-04	YQ-P-05	YQ-P-07	YQ-P-09	YQ-P-11	YQ-P-12
Yeast									
P. kudriavzevii LM-05	-	-	-	-	-	-	-	-	-
S. cerevisiae WQ-11	-	-	-	-	-	-	-	-	-
S. fibuligera SM-03	-	-	-	-	-	-	-	-	-
W. anomalus WQ-10	-	-	-	-	-	-	-	-	-
Filamentous fungi									
A. corymbifera HH-07	-	-	-	-	-	-	-	-	-
R. pusillus HH-05	-	-	-	-	-	-	-	-	-
R. stolonifer SM-02	-	-	-	-	-	-	-	-	-
Gram-positive bacteria									
B. cereus LM-P-05	+	-	-	++	-	+	-	+	-
B. pumilus WQ-P-07	-	-	-	-	-	-	-	-	-
B. subtilis SM-P-07	+	-	-	+	-	+	-	+	-
L. plantarum LM-M-02	-	-	-	-	-	-	-	-	-
M. luteus LM-M-01	-	-	-	-	-	-	-	-	-
S. epidermidis YQ-P-10	++	-	+	++	-	++	+	++	-
Gram-negative bacteria									
A. tropicalis CH-P-02	-	-	-	-	-	-	-	-	-
E. coli SM-V-02	++	-	+	++	-	++	+	++	-
P. pentosaceus YQ-M-10	-	-	-	-	-	-	-	-	-
P. aeruginosa SM-M-04	-	-	-	-	-	-	-	-	-
S. enterica WQ-M-06	++	-	+	++	-	+	++	++	-

-, Not detectable; +, diameter of inhibitory zone smaller than 10 mm; ++, diameter of inhibitory zone between 10 and 15 mm.

the fungal and yeast strains were not inhibited by cell-free supernatants of the nine *B. licheniformis* strains.

DISCUSSION

Currently, the investigation of microbial associations in fermentation foods attracts substantial interest. Recent studies have focused on the preferential growth of particular organisms and the spontaneous succession of microbial communities [7, 20]. During *Daqu* incubation, these microbial associations have been developed and controlled by traditional fermentation technologies. The microbiota changes throughout *Daqu* incubation are important for stabilizing *Daqu* and elucidating the underlying microbiological processes. By understanding these processes, it will become possible to improve the quality and shelf-life of *Daqu*-derived products. To date, however, the microbiota of *Daqu* has received little attention. In particular, the ecology of *Bacillus* throughout *Daqu* incubation has not been reported, despite its reputation as a dominant genus in *Daqu*. The objective of this study was to bridge this knowledge gap using a combination of culture-dependent and culture-independent methods. In general, the *Daqu Bacillus* community is relatively diverse, with 8 species detected by culture-dependent methods, and 7 species (including uncultured *Bacillus* sp.) by culture-independent methods. This is not surprising since *Daqu* is produced by uncontrolled natural fermentation.

This study reveals that *Bacillus* spp. are continuously present throughout *Daqu* incubation, and follow a succession, consistent with the results of Shi *et al.* [22] and Zheng *et al.* [37]. *Bacillus* spp. are versatile and can adapt to diverse pH, temperature, and nutrient conditions [10]. Throughout the incubation, the temperature increases gradually, attaining a maximum of 55°C, as shown in Fig. 1. Such temperatures should have a selective effect on the microbiota, favoring thermotolerant, aerobic endospore-forming bacteria. Because the genus *Bacillus* possesses all of these characteristics, it can persist and rapidly overtake the fungi (which are more numerous at the start of incubation). The antimicrobial activities of *Bacillus* may be another reason why *Bacillus* strains become dominant in *Daqu* [12, 24]. Although *Daqu* is not reported to have strong antimicrobial activity, certain *B. licheniformis* strains isolated from *Daqu* showed antimicrobial activities. From our study, the antimicrobial spectra of the *B. licheniformis* strains are not broad, concentrating on certain bacteria. This may explain why many microorganisms can still be isolated after *Daqu* incubation [28, 37, 38]. Or after living together for a long time, the microorganisms in *Daqu* might have adapted to one another.

In this study, we also observed that viable *Bacillus* cells increase up to the DH stage while the diversity declines. The highest *Bacillus* diversity emerged at the LM stage. The conditions during the LM stage are probably favorable to most of the *Bacillus* species in *Daqu*. As the microenvironment of the fermenting substrate changes, species occurring in low numbers are often out-competed by more abundant species and become undetectable. In this study, *B. licheniformis, B. subtilis, B. pumilus*, and *B. amyloliquefaciens* were identified by both culture-dependent and culture-independent methods. These four species have been reported to exist in other *Daqu* [25, 28, 32]. However, the occurrence of *B. circulans*, *B. anthracis*, *B. benzoevorans*, and *B. foraminis* has not been reported in *Daqu* to date. Unknown *Bacillus* species, indicated as "uncultured," were also detected in our study.

Traditionally, *Bacillus* species have been associated with food spoilage. They reduce the quality and safety of food. More recently, however, they have been linked to fermentation processes in foods such as *tape ketan*, cocoa, *kinema*, and *meju* [10, 17]. *Bacillus* appears to produce thermostable enzymes that degrade cell walls and other polysaccharides, and may be implicated in flavor development and precursors such as pyrazines. Good examples are some African and Asian fermented dishes such as African locust bean, *Thua nao*, and *Bikalga* [9, 19]. The production of these enzymes and aroma precursors is also beneficial to *Daqu* and its derived products [30, 35], although the extent to which *Bacillus* contributes to the texture and flavor development of these products is unknown. Therefore, elucidating the ecology of *Bacillus* in *Daqu* is useful not only to standardize *Daqu* incubation, but also to optimize the quality and safety of its derived products [13].

PCR-DGGE is a powerful technique that can analyze microbial ecology in uncultivated complex microbial communities [8]. Nevertheless, it is subject to misinterpretation arising from incomplete DNA extraction, PCR biases, co-migration, and poor resolution of low abundance microbes. Generally, bacterial populations comprising 1% or more of the total community are detectable by DGGE [31]. This may explain why *B. megaterium*, *B. cereus*, *B. circulans*, and *B. anthracis* were not observed in DGGE, but were found by isolation. These species were likely present in minimal proportions in the samples (<1%). It should also be noted that PCR-DGGE methods detect dead as well as viable cells. Furthermore, the two sampling methods are not the same. The culture-dependent approach measures the germinated spores, determined both by the abundance of spore species and their ability to germinate. By contrast, the culture-independent approach relies on ample DNA extraction of all microorganisms in the sample. These differences will almost certainly contribute to some of the differences found in the diversity of *Bacillus*.

The flavors of *Daqu*-derived products differ with geographical locations [38]. The specific microbial composition of *Daqu* is known to play a key role in the

flavor of its derived products [25, 28, 36, 38]. *B. subtilis* is a dominant microorganism in *Moutai Daqu* and heavily influences the flavor of *Moutai* liquor [23, 26, 33]. In this study, *B. licheniformis* was established as the predominant bacterium in *Daqu* produced by the Shanxi Xinghuacun Fenjiu Distillery Co. Ltd. China (*Fen Daqu*). Liu *et al.* [15] arrived at the same conclusion using culture-dependent methods. *B. licheniformis* is a facultative anaerobe bacterium, able to grow under adverse ecological conditions. It has been implicated in the production of neutral protease in *Fuqu* (another kind of *Qu*) [34]. The bacterium may produce more than 70 metabolites, most of which are flavoring compounds and flavoring precursors used in the liquor industry [27]. More detailed investigations are required to reveal the impact of *B. licheniformis* on the flavor development in *Fen Daqu* and its derived products.

In conclusion, *Bacillus* is dominant and active throughout the seven stages of *Daqu* incubation, and we expect that *Bacillus* species will largely affect the texture and flavor of *Daqu*. *B. licheniformis* was detected at all stages of the incubation by both culture-dependent and culture-independent methods, and was therefore regarded as the persistent dominant *Bacillus* species. We expect this specie to mainly affect the quality of *Daqu.* The impact of *B. licheniformis* on the stabilization, quality and safety of *Daqu* and its derived products will emerge under further investigation.

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