Daqu-
A traditional fermentation starter in China: microbial ecology and functionality

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天道酬勤
地道酬善
人道酬誠
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Abstract

Fermented products have high nutritional value and constitute an important part of the Chinese dietary profile; they are also gaining popularity throughout the world. *Daqu* is a traditional natural fermentation starter culture that has a significant impact on the quality and flavour of Chinese liquor and vinegar.

A review of the literature was conducted focusing on the classification, composition, and manufacture of *Daqu*. The review provided a preliminary understanding of the link between the fermentation process and the characteristics of the final *Daqu* product. Then the occurrence, levels, and diversity of microorganisms were studied in different types of *Daqu* produced by various fermentation processes. The results showed that *Bacillus licheniformis* and *Saccharomycopsis fibuligera* were present in all the tested samples of *Daqu*. Regional comparisons showed *Staphylococcus gallinarum* and *Staphylococcus saprophyticus* in southern *Daqu*. The fungi *Sm. fibuligera* and *Lichtheimia ramosa* were found in low/medium-temperature *Daqu* and *Thermomyces lanuginosus* occurred in high-temperature *Daqu*.

In order to study the functionality of *Daqu* and the contribution of the predominant microorganisms to alcoholic fermentation, the mesophilic and thermophilic bacteria and spores, Enterobacteriaceae, lactic acid bacteria, yeasts, and moulds present in the core and outer portions of *Fen-Daqu* were isolated. The isolates were identified by culture-dependent sequencing of rRNA genes (16S rRNA for bacteria; 18S rRNA, 26S rRNA, and ITS rRNA for fungi). A succession of fungi, lactic acid, and *Bacillus* spp. was associated with prevailing acidity, moisture content, and temperature during *Daqu* fermentation. The predominant species in fermentation were *B. licheniformis*, *Pediococcus pentosaceu*s, *Lactobacillus plantarum*, *Pichia kudriavzevii, Wickerhamomyces anomalus*, *Sacchromyces cerevisiae*, and *Sm. fibuligera*.

One strain of the each of the above-mentioned predominant species, with the highest starch degrading ability and alcohol tolerance, was selected and used in different combinations to perform alcoholic fermentation. Metabolite composition differed significantly between various fermentation trials. *S. cerevisiae* provided superior ethanol

production. *Sm. fibuligera* and *B. licheniformis* provided the amylolytic activity that converted starch and polysaccharides into fermentable sugars. Finally, *W. anomalus* was found to be an important contributor to formation of the liquor aroma.

Understanding the microbial diversity and functional activity, as well as the production dynamics and safety of *Daqu* will enable commercial producers to improve and/or scale-up traditional processes and enhance product quality and safety, thus facilitating entry into international markets.

Chapter 1

General introduction and thesis outline

Chinese fermented foods have received increased attention owing to their specific flavours, nutritional value, and health benefits, and are a major part of the food production system in China. Alcoholic beverages including beer, Chinese liquor, and wine are important indigenous fermented products. In particular, Chinese liquor is an economically vital cultural commodity in China that is enjoyed by consumers at various social occasions. The liquor is typically generated by natural solid-state fermentation (SSF), and the product quality depends primarily on the skills of process operators; in general, the latter have little knowledge of microbiological and biochemical events occurring during the production process. However, non-standardised process parameters often lead to products with inconsistent quality. Several industries have tried to automate their production technology or replace some of the indigenous ingredients, but these attempts have resulted in the loss of unique product flavours due to circumvention of traditional methods. A detailed investigation of the microbiological and biochemical processes involved in the production of traditional fermented foods is therefore necessary for successful commercial-scale production.

1.1 Background

Solid-State Fermentation (SSF)

SSF is a process in which microbial cultures are grown on a solid matrix in the absence of a liquid (aqueous) phase (Barrios-González, 2012). This method has gained an increase in scientific and industrial attention in the past 20 years as a cost-saving measure for the efficient utilisation of agricultural products and waste (Barrios-González, 2012). Several fermented foods and beverages such as soy sauce, *men*, *meju*, and Chinese liquor are traditionally produced by SSF (Kim et al., 2011b; Tanaka et al., 2012; Wang et al., 2008b). SSF can also be applied for the production of enzymes that hydrolyse macromolecules and facilitate fermentation by bacteria and fungi (Singhania et al., 2009). In addition, SSF can provide high yields and can improve the functionalities of a variety of biomolecules including flavours, colourants, preservatives, and sweeteners, which could add value to the fermented products (Couto et al., 2006). SSF-associated indigenous microbiota include different lactic acid bacteria (LAB), *Bacillus* spp., yeast, and fungi

(Nout, 2009). These microorganisms convert substrates into fermentation end-products and also synthesise a variety of aroma compounds. For example, 2,5-dimethylpyrazine and tetramethylpyrazine are important flavour enhancers that are generated by *Bacillus natto* and *Bacillus subtilis*, respectively (Couto et al., 2006). A downside of SSF however, is that microbial growth and community composition are difficult to control; nutrient diffusion, enzyme stability, metabolic activities, and aerobic processes are influenced by various parameters including moisture content, temperature, pH, particle size, aeration, and agitation (Krishna, 2005). A better understanding of the microbiological and biochemical mechanisms underlying SSF would enable better control of the process and assure the quality of the final product.

Production of Chinese liquor (Baijiu)

Chinese liquor (known as *Baijiu* in Chinese) is a potent, distilled spirit containing 40-65% alcohol by volume. The annual production of Chinese liquor has steadily increased in recent years and currently exceeds ten million metric tons annually (Anonymous, 2014). Hundreds of different types of Chinese liquor are produced by various processes in different regions of China, and can be previously classified as strong, light, sauce and miscellaneous, according to their flavour (Shen, 2005), nowadays more flavours such as rice, sesame, *feng*, *chi*, *fuyu*, *yao*, *teyi*, *laobaigan* appear. Representatives of six major Chinese liquor types are shown in Figure 1.1. Traditionally, *Baijiu* contained average ethanol levels of 50-65% v/v, but most products now contain around 40% v/v through the addition of water and adjustment of flavours (Fan et al., 2006).

Techniques used to produce Chinese liquor have a long history and have been passed on through many generations. Chinese liquor is typically produced from grains such as sorghum, wheat, rice, glutinous rice, and maize by a complex SSF process, which comprises (i) material preparation, (ii) grinding and cooking, (iii) mixing with powdered natural starter cultures (*Daqu*) (Figure 1.2), (iv) alcoholic fermentation, and (v) distillation (Hong, 2001). Fresh distillates often have undesirable characteristics such as harsh, green, or raw flavour; therefore, the liquor must be aged for several years to produce the desired balance of aromas through chemical transition, including an acid increase and decrease in ester contents (Zhang et al., 2008).

Figure 1.1 Examples of different types of Chinese liquor (*Baijiu*).

(1) *Daqu* making

Daqu is a specific fermentation starter culture that plays an important role in the production of Chinese liquor and vinegar (Hong, 2001). It not only contributes to starch degradation and alcohol production during the alcoholic fermentation of sorghum, but also strongly impacts the flavour and taste of the final product. *Daqu* contains a diverse microbial population that hydrolyses and ferments substrates, and is cultured under dynamic conditions of temperature and humidity for a few weeks to enable the establishment of the microorganisms that will be functional during the later process stages of alcoholic fermentation (Le et al., 2011; Li et al., 2013).

Figure 1.2 *Daqu* - Chinese traditional fermentation starter blocks (Photo by X.W. Zheng).

Several Asian alcoholic fermentation starters such as *marcha*, *loog-pang*, and *men* have been described (Kishida et al., 2009; Thanh et al., 2008; Tsuyoshi et al., 2005) that contain a mixture of fungi and bacteria in a rice-based tablet. *Daqu* contains similar microorganisms, but is made from a mixture of beans and grains such as pea, wheat, and barley, the latter contributing to the aroma of the final distillate (Cao et al., 2010b; Zhang et al., 2012). *Daqu* contains a combination of yeasts, bacteria, and moulds; its quality highly depends on factors such as ingredient formulation and microbiota composition, which vary by place of origin and confer unique flavours to the fermented liquor (Zhang et al., 2011b). As such, investigations of the microbial diversity and optimum conditions for producing *Daqu* have raised attention of the Chinese liquor production industries.

(2) Alcoholic fermentation

Alcoholic fermentation is typically carried out under anaerobic conditions in earthen jars dug-in the ground or in a cellar (Figure 1.3). About 10-30% *Daqu* powder is added to cooked grains (mainly sorghum) and the mixture is transferred to the jars or cellar where it is left to ferment for about 1 month. The enzymes and chemical components in *Daqu* provide substrates for microbial growth and precursors for the development of the liquor aroma. The entire process consists of polymer degradation, release of carbohydrates, alcohol production, and aroma generation. The main microorganisms involved in fermentation are *Saccharomyces cerevisiae*, non-*Saccharomyces* yeasts, lactic acid bacteria (LAB), and filamentous fungi including *Mucor*, *Absidia*, and *Aspergillus* spp. (Li et al., 2011; Qiao et al., 2004).

Figure 1.3 Alcoholic fermentation from sorghum in earthen jars (left) and in a cellar (right).

(3) Distilling and ageing

Distillation is a key step in the development of aroma in liquor. The impact of distillation depends on four parameters, i.e. steaming time, water content, distillation speed, and porosity of materials (Lai et al., 2005). After distillation is complete, the first distillate containing high concentrations of low-boiling components such as methanol and aldehyde and the last fraction (approximately last half-kilogram of distillate) containing high concentrations of fusel oil (e.g., isoamyl and isobutyl alcohol) and esters of fatty esters (e.g., linoleic acid ethyl ester, ethyl palmitate, oleate), are removed in order to stabilise the aroma and ensure the safety (Wang, 2003).

The collected distillate is aged for several years to develop the bouquet of Chinese liquor, according to an ageing process specific to each type of liquor. In general, the ageing time for sauce-flavour liquor is more than 3 years, while at least 1 year is required for strong- and light-flavour liquors (Zhang, 2003). Ageing plays an essential role in the flavour of liquors, since a variety of aromatic compounds (mainly acids and esters) are balanced during this process through physical changes mainly van der Waals interaction combined with chemical reactions such as reduction-oxidation, esterification, hydrolysis, condensation, decomposition, and the Maillard reaction (Xiong, 2000). Owing to the low storage temperature and dark environment, chemical reactions occur slowly; as such, liquor flavours that require a shorter ageing time are principally induced by physical changes. Intermolecular hydrogen bonding is the most important physical determinant of liquor stability (Zhang, 2003), with longer ageing times leading to stronger van der Waals interactions between ethanol and water; this decreases the spicy taste induced by ethanol, leaving an aroma that is fresh and soft.

Approaches to studying microbial diversity

The analysis of microbial community structure and interspecies interactions are relevant factors in revealing of microbiota function. Standard culture-dependent analyses can detect only between 1% and 10% of all environmental microbiota, suggesting that our knowledge of environmental microbial diversity and function is fairly limited (Muyzer, 1999). Advances in molecular microbiology using techniques such as denaturing gradient gel electrophoresis (DGGE), analysis of single-stranded conformational polymorphisms

(SSCP) and restriction fragment length polymorphisms (RFLP), gene cloning, and next generation DNA sequencing (NGS) have enabled researchers to obtain a broad view of microbial communities present in different environments, including in complex food systems. These approaches along with traditional culture-based methods are currently used to analyse microbial diversity of fermented foods, and the knowledge acquired has been successfully applied to the commercial production of a wide variety of foods and beverages (Erkus et al., 2013).

(1) Culture-dependent approaches

Traditional analyses of microbiota rely on the isolation and enumeration of microorganisms using various types of growth medium (Liu et al., 2010a). Microbial diversity is analysed based on growth, nutrient utilisation, type of energy metabolism, and morphological characteristics. Species identification in foods has been greatly enhanced by sequencing of 16S rRNA (bacteria) and 26S rRNA (fungi) genes. Only 1-10% of environmental microorganisms can be cultured, isolated, and characterised using traditional methods, with the overwhelming majority being uncultivable and thus, inaccessible for conventional analysis (Muyzer et al., 1998). Moreover, culture-dependent approaches can provide only limited information on microbial evolutionary relationships, preventing the accurate classification of existing microorganisms. Nonetheless, active strains can be obtained by this approach, and therefore the development and application of microbial resources still rely on traditional isolation and culture techniques, which are widely used to analyse microbial communities in traditional fermented products both qualitatively and quantitatively (Adewumi et al., 2013; Greppi et al., 2013).

(2) Culture-independent approaches

Culture-independent approaches for evaluating microbial diversity are based on analyses of nucleic acid (DNA and RNA) sequences, for which microbial DNA or RNA is extracted and selected regions amplified by polymerase chain reaction (PCR), with size and composition characterised by gel electrophoresis and nucleotide composition determined by sequencing (Xu, 2006). Three standard culture-independent approaches are the construction of environmental DNA clone libraries (Riesenfeld et al., 2004), DGGE- or temperature gradient gel electrophoresis (TGGE)-based community fingerprinting (Ercolini, 2004), and

NGS-based community profiling (Li et al., 2013), all of which have been applied for the investigation of microbial diversity in fermented food products.

Constructing clone libraries from phylogenetic markers such as 16S (bacteria) or 26S (fungi) rRNA genes is currently the most widely used method for assessing microbial community composition and diversity (Green et al., 2012; Timmis et al., 2010). In gene cloning, amplified target genes are ligated into a plasmid vector that is introduced into competent cells (typically *Escherichia coli*). Clones derived from individual transformant cells are used to construct a clone library and for subsequent sequence analysis. Although library construction is labour-intensive and costly, sequence analyses using libraries are highly efficient in providing information about the microbial composition of food products.

DGGE and TGGE are techniques involving the sequence-specific separation of PCR-derived rRNA gene amplicons on polyacrylamide gels containing a linearly increasing concentration gradient of denaturant (urea and formamide) or temperature, respectively (Muyzer et al., 1998). In a DGGE gel, double-stranded DNA fragments are subjected to increasing denaturing conditions that induce partial melting in discrete regions termed melting domains, starting from the domain with the lowest melting temperature (which is dictated by nucleotide sequence). This creates denatured DNA molecules that have reduced mobility on acrylamide gels. Therefore, DNA fragments of the same size but with different base compositions behave distinctly in the denaturing gradient. DGGE analysis can be applied to the monitoring of microbial fermentation during food production, assessment of microbial communities in food products, and analysis of food microbial community dynamics in response to environmental changes (Adewumi et al., 2013; Andorra et al., 2010; Chen et al., 2011; Guan et al., 2012).

A more recently developed culture-independent approach is NGS, which has replaced Sanger-based sequencing as the mainstream sequencing technology. NGS has been applied to investigations of microbial diversity in environmental, medical as well as in food-related ecosystems (Claesson et al., 2010; Li et al., 2013), as it allows a high-throughput analysis of materials to generate a massive amount of raw sequencing data in a short time at a relatively low cost. The use of NGS in metagenomics research requires significant computational resources as well as experienced bioinformaticians to perform data analyses

(Scholz et al., 2012). Moreover, it requires careful consideration of the research objective and information demand in order to make efficient use of the large quantities of data that are obtained, which concern a broad range of biological phenomena (genetic variation, RNA expression, protein-DNA interaction, chromosome conformation, etc.).

Microbial activity, environment, and food product characteristics

Production of fermented foods creates an ecological environment in which microorganisms are subjected to the influence of various internal (pH, water activity, nutrient composition) and external (temperature, gases, contaminating microbes) factors. Environmental factors can directly influence microbial community structure and activities, which may in turn affect food quality (Figure 1.4). During the *Daqu* making process, LAB produce several types of natural antimicrobials, including organic acids (such as lactic, acetic, formic, phenyllactic, and caproic acids), carbon dioxide, hydrogen peroxide, ethanol, and bacteriocins, which suppress the growth of competing bacteria such as *Bacillus* spp. (Liu, 2011). LAB-generated organic acids create an acidic environment and directly influence the pH of the *Daqu* product (Hai et al., 2014). On the other hand, changes in pH can also adversely affect the growth of LAB (Katina et al., 2002), which have an optimal range for growth between pH 4.3 and 6.5 (Adamberg et al., 2003; Yan et al., 2002). Meanwhile, a reduced production of acetic and lactic acids can negatively impact the flavour of the final liquor (Cao et al., 2010a). Mesophilic bacteria dominate the first phase of fermentation and are followed by LAB during *Daqu* production, resulting in an increase in *Daqu* acidity (Zhang et al., 2011b). Therefore, interactions between chemical, physical, and microbial components of *Daqu* may improve the final sensory characteristics of Chinese liquor by shortening fermentation time and/or enhancing microbial growth via the control of physical parameters during *Daqu* production.

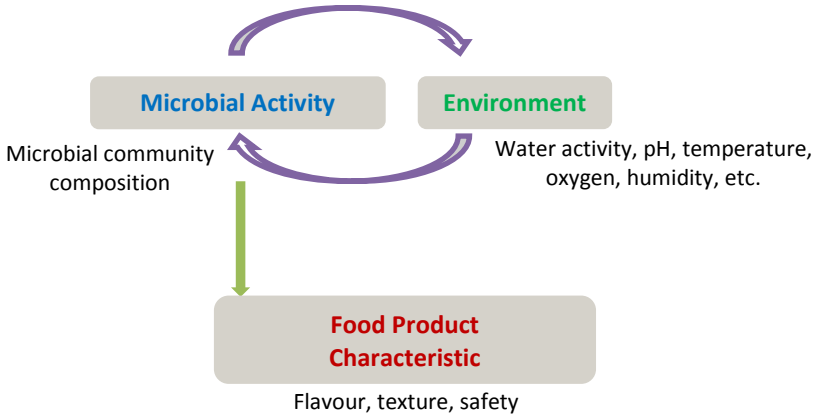

Figure 1.4 Relationship between microbial activity, environment, and food product characteristics.

1.2 Research objectives and thesis outline

The main objectives of the study described in this thesis are: (1) to gain insight into the biodiversity and population dynamics of the microbial community in *Daqu* and their relationship to environmental conditions; and (2) to determine the effects of the major *Daqu* microbiota on subsequent sorghum alcoholic fermentation, in order to generate a knowledge-base for the improvement of *Daqu* production with prospects for quality development of Chinese liquor.

Information on traditional *Daqu* fermentation, including classification and microbiological aspects, is presented in **Chapter 2**. During preliminary field work, the production of different types of *Daqu* was observed, and representative starter samples were collected from leading producers in China and characterised according to their microbial composition using culture-independent cloning methods in conjunction with multivariate statistical analysis. Representative biomarkers for different types of *Daqu* were identified (**Chapter 3**). Based on specific production characteristics, *Fen-Daqu* was selected as the starter for subsequent culture-dependent investigation. The microbial diversity of *Fen-Daqu* was compared across different *Daqu* fractions, and bacterial, yeast, and mould species were isolated (**Chapter 4**). The environmental and microbial changes during *Fen-Daqu* production were also investigated, and the link between environmental parameters and microbial communities are discussed in **Chapter 5**. The microbial

composition in the successive stages of *Daqu* making processes and in the final products was assessed, and the most abundant bacterial and yeast species were identified. **Chapter 6** describes the screening and characterisation of functional bacteria and yeasts for the selection of starter cultures to carry out laboratory-scale alcoholic fermentation using sorghum. The contribution of each microbial species is discussed. Finally, **Chapter 7** is a general discussion of *Daqu* types and their respective contributions to alcoholic fermentation as well as proposed improvements to the *Daqu* manufacturing process.

Chapter 2

Review: *Daqu* - A traditional Chinese fermentation starter

Abstract

Chinese liquor is one of the world's oldest distilled alcoholic beverages, and it is typically obtained with the use of *Daqu* fermentation starters. *Daqu* is a saccharifying and fermenting agent, having a significant impact on the flavour of the product. *Daqu* can be categorized according to maximum incubation temperatures (high, medium and low) and flavour (sauce, strong, light and miscellaneous). Most *Daqu* are prepared by solid-state fermentation from wheat, barley and/or peas by ingredients formulation, grinding and mixing, shaping, incubation, and maturation. Although there is a wealth of artisanal experience in the production of a range of different types of *Daqu*, the scientific knowledge base - including the microbiota, their enzymes and their metabolic activities - needs further development. *Daqu* as a specific alcoholic starter is compared with other Asian amylolytic fermentation starters in terms of microbial diversity and function. Filamentous fungi (*Rhizopus*, *Rhizomucor*, *Aspergillus*, and other genera), yeasts (*Saccharomyces*, *Candida*, *Hansenula*, and other genera) and bacteria (acetic acid bacteria, lactic acid bacteria and *Bacillus* spp.), are considered to be the functional microbiota, responsible for the formation of a range of lytic enzymes, formation of substrates for alcoholic fermentation and formation of flavour compounds. However, the knowledge about the microbiota composition and their function is still fragmentary information, so further research is required to establish the functionality and growth kinetics of microbiota in diverse types of *Daqu*.

Zheng, X.W., Tabrizi, M.R., Nout, M.J.R., Han, B.Z., 2011. *Daqu* - a traditional Chinese liquor fermentation starter. Journal of the Institute of Brewing 117, 82-90.

2.1 Introduction

Alcoholic fermented products play an important role in the quality of Chinese life and culture, and have a long history. The annual production of Chinese liquor in 2008 has been estimated at about 5 million metric tons per year (Anonymous, 2008). Chinese liquor is typically obtained from cereals such as sorghum by complex fermentation processes using natural mixed culture starters (Fan et al., 2007).

Already over 20 years ago, Hesseltine et al. (1988) mentioned three major categories of amylolytic starters used in Asia for alcoholic fermentation, namely (1) koji, pure cultures of *Aspergillus sojae* and *Aspergillus oryzae*, used for soya sauce and miso production; (2) large compact cakes based on whole-wheat flour, fermented for about 4 weeks, containing yeasts, *Rhizopus* and *Absidia* spp., used for the "*kao-liang*" process of alcohol fermentation; and (3) mixed cultures of yeasts, bacteria and fungi, in the form of flattened or round balls, compact in texture and dry. These are allowed to ferment for a short time, dried and used to make alcohol or fermented foods from starchy materials such as rice or cassava.

During the recent past, most of the attention in scientific publications has been given to categories 1 and 3. Koji (category 1) production and its use in soy sauce manufacture was reviewed previously (Nout et al., 2002), as well as a number of examples of category 3 starters, such as *ragi* (Nout, 1992), *men* (Dung et al., 2006), *bubod* (Sakai et al., 1985), murcha (Tamang et al., 1995), and *nuruk* (Lee, 2001). In contrast, category 2 starters have been underreported in the international literature. This is precisely the type of product that is the subject of our present review. Whereas the name "*kao-liang*" used earlier (Hesseltine et al., 1988) seems to be out of use presently, Chinese people know this starter as "*Daqu*". The purpose of this review is to provide a "state-of-the-art" of the present *Daqu* making process, microbiology and biochemistry.

In order to better understand the complex Chinese terminology, a short explanation of the major terms is provided below. The key words include "*Qu*" (moulded cereals), "*Jiuqu*" (moulded cereals used as an alcoholic fermentation starter), and "*Daqu*" (moulded cereals used as an alcoholic fermentation starter, obtained through natural fermentation).

In more detail, "*Qu*" [tsju:] written in hieroglyphics, was reported to have been discovered accidentally through natural fermentation. "*Qu*" are moulded cereals which are

a source of enzymes necessary for the degradation of carbohydrates and proteins in grains, and supply a portion of the substrate for fermentation as well. "*Jiuqu*" [djū, tsju:] as a Chinese word came from the word "*Jiu*" [djū] or "alcoholic beverage", and "*Qu*" as the traditional fermentation starter. Based on their ingredients and technological application, "*Jiuqu*" can be classified into three categories, which are *Daqu*, *Xiaoqu* and *Fuqu* (Hong, 2001).

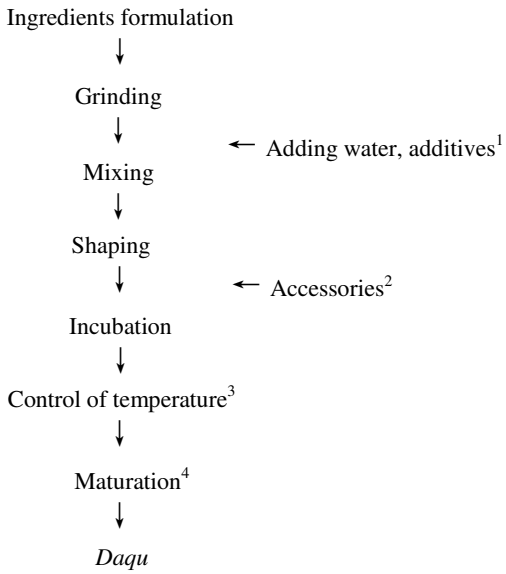

Figure 2.1 The production process of *Daqu*.

[1] Some *Daqu* such as *Moutai-Daqu* use previous Qu as additives; [2] Accessories such as rush mats, bamboo, etc. are used as covers to control the temperature; [3]Involves low temperature incubation phase, high temperature converting phase and aroma creating phase; [4] Maturation aims at achieving spatial equilibrium of moisture, acidity, enzyme activities and microbiota.

Daqu is the type of grain *Qu*, which is made from raw wheat, barley and/or pea. It is a natural fermentation starter, especially for distilled liquor and traditional vinegar production. Powdered *Daqu* are used to inoculate cooked cereals (mainly sorghum) and this mixture is then fermented in sealed jars or cellar for about one month in order to carry on distillation. On the other hand, *Xiaoqu* and *Fuqu* are inoculated starters, consisting of cereal flour or bran that was inoculated with mother culture or moulds, respectively.

In the development of different *Qu*, *Daqu* becomes more and more important, because in liquor production it has more functions than just providing inocula. The exact origin(s) of manufacture of *Daqu* is difficult to establish, but it can be safely assumed to date back to the Han Dynasty (221 BC to 207 AD) (Needham, 2000; Qin, 2000). Nowadays, almost all famous liquors in China are made with *Daqu*. *Moutai* liquor, *Luzhou* liquor, *Fen*-liquor and *Xifeng* liquor are the representatives of four most famous and typical Chinese liquor flavours (sauce-flavour, strong-flavour, light-flavour, and miscellaneous-flavour, respectively), which are all fermented using *Daqu* as a fermentation starter.

The shape, microbial composition and function of *Daqu* are very different from those of Asian amylolytic starters of categories 1 and 3 according to Hesseltine et al. (1988). In particular, *Daqu* contains (a) cereals such as wheat, barley plus significant amounts of peas, (b) a mixed microflora of fungi and bacteria, (c) microbial enzymes and metabolites, and (d) an important flavour note that will contribute to the aroma of the final distillate (Fu, 2004; Hong, 2001; Kang, 1991; Wang et al., 2004).

Another difference with categories 1 and 3 amylolytic starters is that *Daqu* also functions as the initial fermentation substrate for liquor making, similar to some other solid fermentation products, such as sourdough bread (De Vuyst et al., 2005; Gänzle et al., 2008). *Daqu* is thus not only a source of inoculum, but it also represents about 20% of the substrate for alcoholic fermentation. For this reason, considerable quantities of *Daqu* powder are required in the fermentation of Chinese liquor. For example, in *Fen*-liquor fermentation, *Daqu* represents 10-15% of the total substrate, while for *Luzhou* liquor, even more (20-25%) *Daqu* is added.

2.2 Classification and composition of *Daqu*

Classification of Daqu

Different types of *Daqu* can be distinguished based on their maximum incubation temperatures and their flavour characteristics.

(1) Temperature control characteristics

Daqu production involves specific time-temperature control schemes resulting in a succession of microorganisms. According to the maximum incubation temperature,

achieved as a natural result of metabolism, the three different types of *Daqu* can be distinguished (Table 2.1). They are as follows. (i) High-temperature *Daqu* represented by *Moutai* and *Site* liquor *Daqu* (Xiong, 2005c): Maximum temperatures reached during the incubation period range between 60 and 70°C; (ii) Medium-temperature *Daqu* represented by *Wuliangye and Luzhou* liquor *Daqu* (Xiong, 2005b): Maximum temperatures reached during the incubation period range between 50 and 60°C; (iii) Low-temperature *Daqu* represented by *Fen*-liquor and *Erguotou* liquor (Xiong, 2005a). Maximum temperatures reached during incubation period range between 40 and 50°C.

(2) Flavour characteristics

According to their flavour, *Daqu* can be classified into four major types. (i) Sauce-flavour *Daqu* such as *Moutai* liquor *Daqu* (Xiong, 2005a) which gives a liquor with a flavour reminding of soy sauce, full bodied, and with a long lasting aroma. A major representative aroma compound of sauce flavour liquor is tetramethylpyrazine occurring at levels of 3000-5000 mg/l, which is higher than in other types of liquor (Fan et al., 2007), (ii) Strong-flavour *Daqu*. The liquor produced by this kind of *Daqu* such as *Luzhou* liquor has a fragrant flavour, soft mouthfeel, and "endless" aftertaste. The representative aroma compounds are predominantly ethyl hexanoate, in harmonious balance with ethyl lactate, ethyl acetate and ethyl butanoate (Xu, 1991); (iii) Light-flavour *Daqu*. The liquor such as *Fen*-liquor (Xiong, 2005a) produced with this type of *Daqu* is known by its pure and mild flavour, mellow sweetness and refreshing aftertaste. The representative aroma compounds in light flavour liquor are mainly ethyl acetate, in balance with considerable levels of ethyl lactate; (iv) Miscellaneous-flavour *Daqu*. The liquor produced with this type of *Daqu* can have sensory characteristics ranging between those of sauce-flavour *Daqu* and strong-flavour *Daqu*. The representative aroma compounds in corresponding miscellaneous flavour liquors are heptanoic acid, ethyl heptanoate, isoamyl acetate, 2-octanone, isobutyric acid, and butyric acid.

Composition of Daqu

(1) Microbiological aspects

The large number of liquor brewing enterprises countrywide in China, with their unique ecological environments and diverse manufacturing procedures result in typical

"home microbiota" with a large diversity of microorganisms in *Daqu*. In general, four categories of microorganisms occur in *Daqu*: filamentous fungi (moulds), yeasts, bacteria and actinomycetes (Wu, 2004; Yang et al., 2007; Zhang, 1999). Table 2.1 summarizes the groups of microorganisms that have been isolated and reported from different types of *Daqu.* Of the filamentous fungi, the role of *A. oryzae* and *A. sojae* in koji has been studied extensively (Machida et al., 2008; Wicklow et al., 2007) and their production of proteolytic, and other lytic enzymes has been linked to the transformation of insoluble soya bean and wheat compounds into water-soluble peptides, sugars, free amino acids and other degradation products that constitute soy sauce. Also in *Daqu*, *Aspergillus* spp. have been reported in representatives of high (Huang et al., 1993; Wang et al., 2008a), medium (Wu et al., 2009b; Yao et al., 2005a) and low-temperature (Xiong, 2005a) types. Few identifications have been published except in one type (Huang et al., 1993) where *Aspergillus flavus*, *A. oryzae* and *Aspergillus niger* were reported. According to Wang et al. (2008a), *Aspergillus* spp. Would contribute to the saccharification of starch. The fungi mentioned by Hesseltine et al. (1988) for category 2 starters included yeasts, *Absidia* and *Rhizopus* spp. Indeed these were reported to some extent in *Daqu*. Of the yeasts, *Candida* (Huang et al., 1993; Leimena, 2008; Yao et al., 2005a)*, Citeromyces, Debaryomyces, Oosporidium* (Wu et al., 2009b)*, Endomycopsis, Hansenula* (Xiong, 2005b), *Hyphopichia*, *Issatchenkia, Zygosaccharomyces* (Jiang, 2004; Jiang et al., 2003)*, Pichia* (Li et al., 2005; Wu et al., 2009b), *Rhodotorula* (Leimena, 2008)*,, Saccharomyces* (Huang et al., 1993; Leimena, 2008; Wu et al., 2009b) and *Torulopsis* (Huang et al., 1993) were encountered, but there is no evidence to date that indicates the continuous presence of one or more of these genera. In some types of *Daqu*, data about yeasts are unavailable. Concerning the filamentous fungi, on the one hand, the genera *Absidia* (Leimena, 2008; Wu et al., 2007; Xiong, 2005a; Yao et al., 2005a) and *Rhizopus* (Huang et al., 1993; Wang et al., 2008a; Xiong, 2005a), mentioned by Hesseltine et al. (1988) were encountered in *Daqu* but they do not seem to represent a constant factor. In the same way, other filamentous fungi such as *Aspergillus* (Huang et al., 1993; Leimena, 2008; Wang et al., 2008b; Wu et al., 2009b), *Gilmaniella* (Wu et al., 2007)*, Monascus, Penicillum, Mucor* (Huang et al., 1993; Leimena, 2008; Wang et al., 2008a; Yao et al., 2005a), *Trichoderma* (Huang et al., 1993), and

Rhizomucor (Leimena, 2008), were encountered in *Daqu*, but no single genus was found to be always present. *Monascus* spp. Are associated with ester formation (Wang et al., 2008a), *Trichoderma* spp. With degradation of cellulose and starch (Wang et al., 2008a) and *Rhizopus* spp. With formation of volatiles (Wang et al., 2008a). *Penicillium* spp. Are considered to be undesirable contaminants as they can interfere with *Daqu* quality by inhibiting the growth of other beneficial micro-organisms (Shen, 2001b). Clearly, the data available to date are quite fragmentary and more detailed studies will be required to fill the gaps and to get more insight into characteristic fungal communities. In certain high- and low-temperature *Daqu*, *Actinomycetes* were encountered (Li et al., 2009; Wu et al., 1993). Their possible role in the process is not yet known. In category 3 amylolytic starters, Hesseltine et al. (1988) found it remarkable that in each starter at least one species of *Mucor, Rhizopus* and *Amylomyces* was present. Yeast counts were mostly in the range of 7-8 log CFU/g, as was confirmed by subsequent researchers. However, available data on *Daqu* do not mention the presence of *Amylomyces* spp..

In *Daqu* the presence of bacteria has been reported by several investigators, and on the basis of the limited information it appears that acetic acid bacteria, lactic acid bacteria and *Bacillus* spp. (Li, 2008; Li et al., 2009; Li et al., 2005; Wang et al., 2008a) are predominant and that bacterial loads may vary between 5 and 8 log CFU/g (Table 2.1). Our own preliminary data on *Erguotou-Daqu* indicate the presence of 19pprox.. 8 log CFU/g, mainly *B. licheniformis* (Leimena, 2008). The interesting aspect of the presence of high levels of *Bacillus* spp. Is that their functional role in other fermented products such as cocoa (Schwan et al., 2004), *kinema* (Sakar et al., 1994) and *dawadawa* (Meerak et al., 2008) is related to the degradation of cell walls and other polysaccharides and proteins, as well as the formation of flavours and precursors such as pyrazines (Larroche et al., 1999). *Bacillus* spp. Were reported to be a continuous factor in *Daqu*. They can contribute to the evolution of flavour (Fan et al., 2007) and enzyme activities such as amylases and proteinases (Wang et al., 2002) needed in the fermentation of cooked sorghum for alcoholic fermentation.

Until recently, most Chinese publications relied on culture-dependent microbiological analysis using selective media (i.e., with antibiotics (Yao et al., 2005a), sodium desoxycholate (Xu et al., 2002), cycloheximide (Lei et al., 2006), ampicillin (Mu et al.,

2004), or nystatin (Mu et al., 2004)). These revealed the presence of *Lactobacillus*, *Acetobacter* and *Bacillus* spp., but did not lead to species identifications. Mycological media in combination with morphological, physiological and biochemical methods for characterization, such as temperature for growth (Li, 2004) and assimilation of carbon and nitrogen sources (Guo, 2006) revealed the occurrence of fungi, mainly *Aspergilus*, *Rhizopus*, *Mucor*, *Penicillium*, *Monascus*. And *Absidia* spp. (Shen, 2001b; Wan, 2004). In addition, some *Thermoactinomycetes* were reported to be present in few types of *Daqu* at levels of 5-8 log CFU/g *Daqu.* These are typical soil microorganisms and are not considered to be relevant for the production of liquor (Li et al., 2009; Wu et al., 1993).

Modern culture-independent techniques have recently been used to study the microbial diversity in *Zaopei* (an intermediate semi-finished product during Chinese liquor fermentation) (Zhang et al., 2005; Zhang et al., 2007a) and fermented grain (Wang et al., 2008b), but only few such studies were undertaken for *Daqu* to date (Leimena, 2008; Li et al., 2009). Xie et al. (2007) compared fungal diversity in wheat *Qu* (which differs from *Daqu* and is used for the production of Shaoxing rice wine) using both culture-dependent and molecular-based methods. ITS sequences of RISA (Ribosomal Intergenic Spacer Analysis) bands revealed the presence of 13 fungal species, many of which were not detected from plate cultures. On the other hand, some cultured species were not detected by RISA. This shows the importance of developing an integrated approach to gain a better understanding and better coverage of microbial communities in complex food systems. We expect that polyphasic studies of *Daqu* microbiota will provide more detailed species and strain level data on the functional microbiota of different types of *Daqu*.

(2) Enzymatic aspects

The microbiota of *Daqu* (e.g., *Rhizopus*, *Aspergillus* and *Monascus*) (Fan et al., 2000) produce a range of enzymes in *Daqu* such as α-amylase, β-amylase, glucoamylase and proteases. It is likely that in *Daqu*, *Bacillus* spp. Produce amylases as well. The function of amylases is to degrade starch into dextrins or maltose, and further to glucose (Wan, 2004). Some yeasts have autolysis property, which can result in the accumulation of nutrients for microbial growth. Some peptides and amino acids can influence the formation of aroma and even the flavour of the final liquor (Wan, 2004). Only acid proteases have been reported to

be present in *Daqu,* particularly those producing single amino acids as their final product (Shen, 2001b). This type of protease is active only after the lysis of yeast cells, i.e., at the end of the alcoholic fermentation, which contributes to adequate aroma compounds for the final taste of Chinese liquor (Shen, 2001b).

(3) Chemical aspects

Daqu is mostly made from wheat, barley and/or peas, which are significant sources of carbohydrates, crude protein, crude fat, minerals, and amino acids (Men et al., 1995; Shi et al., 1996). Because the proportion of these raw materials can differ for different types of *Daqu*, the chemical composition shows certain variability. The biggest fraction in *Daqu* is starch, which represents about 40-60% of the dry matter. That is also the reason why *Daqu* is popularly referred to as a "starch based starter". With an additional protein fraction representing around 13-20%, the ingredients offer a good source of nutrients and substrate for the growth of microorganisms during liquor fermentation. Although the proximate chemical composition of *Daqu* has been analysed, little information is available about the role of specific microbiota on the chemical compounds present. Recently a non-targeted metabolomics approach using high resolution ^{1}H nuclear magnetic resonance (NMR) was taken in order to distinguish representative compounds of well-known flavour types of *Daqu* (Wu et al., 2009a). It was observed that this spectroscopy combined with principal component analysis, enabled the distinction of flavour types and specific biomarkers were identified for each of these. Higher levels of glycerol, malate, acetate and N-acetylglutamine are biomarkers for light-flavour *Daqu*; higher levels of mannitol, betaine, trimethylamine and pyroglutamate are biomarkers for strong-flavour *Daqu*; and higher levels of lactate, isoleucine, leucine, isovalerate and valine are biomarkers for sauce-flavour *Daqu*.

Table 2.1 Microbiota isolated from different types of *Daqu*

Microorganisms	High-temperature *Daqu* (60-70°C)			Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)	
	Moutai	*Site*	*Lang*	*Wuliangye*	*Luzhou*	*Xufang*	*Fen*	*Erguotou*
Moulds (total viable counts)	[7.0](Wang et al., 2008a)				[6.4](Yao et al., 2005a)	[6.7], [6.1]*(Shi et al., 2001)	$[6.2]_a$; $[6.7]_b$; $[6.1]_c$ (Xiong, 2005a)	
Absidia spp.	[P](Wu et al., 2007)				[6.7](Yao et al., 2005a)		$[4.8]_a$; $[4.8]_b$; $[5.0]_c$ (Li et al., 2005)	
Absidia corymbifera								[P](Leimena, 2008)
Aspergillus spp.	[6.9](Wang et al., 2008a)	[6.5](Huang et al., 1993)			[5.2](Wu et al., 2009b; Yao et al., 2005a)		$[4.2]_a$; $[4.4]_b$; $[3.8]_c$ (Li et al., 2005)	
Aspergillus flavus		[5.5](Huang et al., 1993)						
Aspergillus oryzae		[5.6](Huang et al., 1993)						
Aspergillus niger		[4.6](Huang et al., 1993)						
Gilmaniella spp.	[P](Wu et al., 2007)							
Monascus spp.	[P](Jiang, 2004; Jiang et al., 2003)	[5.7](Huang et al., 1993)			[6.0](Yao et al., 2005a)			
Mucor spp.	[6.3](Wang et al., 2008a)	[7.7](Huang et al., 1993)			$[6.7]_{§§}$(Yao et al., 2005a)			
Mucor circinelloides								[P](Leimena, 2008)

Table 2.1 Microbiota isolated from different types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)			Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)	
	Moutai	*Site*	*Lang*	*Wuliangye*	*Luzhou*	*Xufang*	*Fen*	*Erguotou*
Penicillum spp.	[P](Jiang, 2004; Jiang et al., 2003)				[5.9](Yao et al., 2005a)			
Trichoderma spp.		[3.4](Huang et al., 1993)						
Rhizopus spp.	[6.0](Wang et al., 2008a)	[2.6](Huang et al., 1993)					$[3.1]_a$; $[3.2]_b$; $[2.9]_c$(Li et al., 2005)	
Rhizomucor pusillus								[P](Leimena, 2008)
Rhizomucor variabilis								[P](Leimena, 2008)
Yeasts (total viable count)	[6.0](Wang et al., 2008a)				[5.4](Tang et al., 2005)	[6.0](Shi et al., 2001); [5.8]*(Shi et al., 2001)	$[5.9]_a$(Li et al., 2009); $[5.9]_b$(Li et al., 2009); $[5.8]_c$(Li et al., 2009)	[6.7]§(Leimena, 2008)
Candida spp.					[5.8](Yao et al., 2005a)			
C. kunwiensis								[P](Leimena, 2008)
C. utilis		[6.5](Huang et al., 1993)						
Citeromyces spp.					[P](Wu et al., 2009b)			
Debaryomyces spp.					[P](Wu et al., 2009b)			
Endomycopsis spp.							$[3.7]_a$(Xiong, 2005a); $[3.7]_b$(Xiong, 2005a); $[3.6]_c$(Xiong, 2005a)	

Table 2.1 Microbiota isolated from different types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)			Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)	
	Moutai	*Site*	*Lang*	*Wuliangye*	*Luzhou*	*Xufang*	*Fen*	*Erguotou*
Hansenula anomala		[6.3](Huang et al., 1993)						
Hyphichia burtoniia	[P](Jiang, 2004; Jiang et al., 2003)							
Issatchenkia orientalis	[P](Jiang, 2004; Jiang et al., 2003)							
I. scutulata varaxingae	[P](Jiang, 2004; Jiang et al., 2003)							
Oosporidium spp.					[P](Wu et al., 2009b)			
Pichia spp.					[P](Wu et al., 2009b)		[P](Li et al., 2005)	
Rhodotorula colostri								[P](Leimena, 2008)
Saccharomyces spp.					[P](Wu et al., 2009b)			
S. bayanus×cerevisiae hybrid								[P](Leimena, 2008)
S. cerevisiae var. ellipsoideus		[6.4](Huang et al., 1993)						
Saccharomycopsis fibuligera								[P](Leimena, 2008)
Torulopsis glabrata		[6.4](Huang et al., 1993)						

Table 2.1 Microbiota isolated from different types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)			Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)	
	Moutai	*Site*	*Lang*	*Wuliangye*	*Luzhou*	*Xufang*	*Fen*	*Erguotou*
Lachancea cidri (Zygosaccharomyces cidri)	[P] (Jiang, 2004; Jiang et al., 2003)							
Bacteria (Total viable count)	[7.8] (Wang et al., 2008a)		[7.9] (Wu, 2004)	[7.9] (Wu, 2004)	[5.9] (Yao et al., 2005a)	[6.2] (Shi et al., 2001) [5.0]* (Shi et al., 2001)	$[9.3]_a$ (Li et al., 2009); $[9.4]_b$ (Li et al., 2009); $[9.5]_c$ (Li et al., 2009)	
Acetic acid bacteria		[6.5] (Huang et al., 1993)	[4.1] (Wu, 2004)	[5.9] (Wu, 2004); [P]† (Wang et al., 2008b)	[P] (Yao et al., 2005a)		$[5.8]_a$ (Li et al., 2009); $[5.6]_b$ (Li et al., 2009); $[5.6]_c$ (Li et al., 2009)	
Bacillus spp.	[7.60] (Wang et al., 2008a)	[6.8] (Huang et al., 1993)	[7.3] (Wu, 2004)	[6.5] (Wu, 2004); [P]† (Wang et al., 2008b)	[5.7] (Tang et al., 2005)	[6.3] (Shi et al., 2001); [5.7]* (Shi et al., 2001)	$[9.5]_a$ (Li et al., 2009); $[9.3]_b$ (Li et al., 2009); $[9.8]_c$ (Li et al., 2009)	
B. amyloliquefaciens	[P] (Jiang, 2004; Jiang et al., 2003)						[P] (Li, 2008)	
B. cereus							[P] (Li, 2008)	
B. coagulans	[P] (Jiang, 2004; Jiang et al., 2003)							
B. firmus	[P] (Jiang, 2004; Jiang et al., 2003)							
B. licheniformis	[P] (Jiang, 2004; Jiang et al., 2003)						[P] (Li, 2008)	[P] (Leimena, 2008)

Table 2.1 Microbiota isolated from different types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)			Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)	
	Moutai	*Site*	*Lang*	*Wuliangye*	*Luzhou*	*Xufang*	*Fen*	*Erguotou*
B. pumilus							[P](Li, 2008)	
B. stearothermophilus	[P](Jiang, 2004; Jiang et al., 2003)							
B. subtilis	[P] (Jiang, 2004; Jiang et al., 2003)						[P](Li, 2008)	
B. thermoglucoseidasuis	[P] (Jiang, 2004; Jiang et al., 2003)							
B. thuringiensis							[P](Li, 2008)	
Enterobacteriaceae								[5.3](Leimena, 2008)
Lactic acid bacteria		[6.7](Huang et al., 1993)	[7.6] (Wu, 2004)	[P]†(Wang et al., 2008b)	[P](Wu et al., 2009b; Yao et al., 2005a)		$[8.3]_a$(Li et al., 2009); $[7.7]_b$(Li, 2008); $[8.3]_c$(Li, 2008)	
L. acetotolerans				[P]†(Wang et al., 2008b)				
L. manihotivorans				[P]†(Wang et al., 2008b)				
L. panis				[P]†(Wang et al., 2008b)				

[ ] = log CFU/g; a: A type of *Fen Daqu*, named *Hongxin Daqu;* b: A type of *Fen Daqu*, named *Qingcha Daqu;* c: A type of *Fen Daqu*, named *Houhuo Daqu;* [P] Presence confirmed by identification of isolated pure cultures; $[P]_†$ Presence confirmed in similar product, by molecular identification; ∗ Identified from a very similar product; § Yeasts & Moulds counted together; §§ *Mucor* & *Rhizopus* spp. counted together.

2.3 Manufacturing process of *Daqu*

Common procedure for Daqu making

The present production of *Daqu* has evolved from a simple artisanal scale to a large scale industrial process. Whereas in the past, liquor producers purchased starter from a manufacturer that used stone mills, nowadays liquor factories produce their own *Daqu* using mechanical milling operations. Nevertheless, the principle of the process has remained the same and can be summarized in five steps (Figure 2.1). These are (i) ingredients formulation, (ii) grinding and mixing, (iii) shaping, (iv) incubation, and (v) maturation. The steps are discussed below.

Ingredients formulation: Most of the high-temperature *Daqu* is made from pure wheat, while the medium-temperature *Daqu* is made with a blend of barley and peas.

Grinding and mixing: The purpose of grinding is to break the grains to release starch with the aim of increasing water absorption to obtain a desirable cohesion of the mass. The main purpose of mixing with water is to obtain a homogenous texture and dispersion of the added water and additives in all parts of the substrate.

Shaping: The wetted material is transferred to a molding press and formed to a brick weighing approximately 1.5-4.5 kg each, with either flat surfaces, or with one end in a convex shape.

Incubation: This step of *Daqu* processing is the period during which the incubation temperature needs to be controlled. Specific incubation conditions for *Daqu* vary according to the *Daqu* type to be produced. The three-stage principle used for strong- and sauce-flavour *Daqu* consists of (a) a low temperature incubation phase, (b) a high temperature "converting" phase and (c) an aroma creating phase. To adjust the temperature, for some types such as light-flavour *Daqu*, three different arrangements of stacking of the *Daqu* bricks are used to achieve adequate ventilation and maximum yield, as shown in Figure 2.2. During the first day of incubation, *Daqu* is still quite soft and thus may only be stacked to a maximum of 3 layers to avoid deformation and to allow good ventilation (Figure 2.2A). Next, when the *Daqu* texture becomes harder, and temperature control needs to be optimized, arrangements such as shown in Figures 2.2B and 2.2C can be used according to

the requirements for the different incubation phases. The basis for control for these different phases is as follows:

(a) Low temperature incubation phase: The aim of this phase is to activate initial microbial growth and to allow the temperature to increase gradually, attaining 30-40ºC in 3-5 days. The initial 24-48 h is considered to be a crucial time for establishing the structure of *Daqu*'s microbial community; pioneer microorganisms such as fungi start to colonize and mycelium will spread over the surface of *Daqu*.

(b) High temperature "converting" phase: The goal of this phase is to accumulate flavour compounds by the metabolic conversion of amino acids. This phase is characterized by a controlled and gradual increase of the temperature at a rate of 5-10ºC/day, while maintaining the relative humidity at 90%, with the aim of increasing the microbial metabolic rate and accumulating CO_2 in the product. After finally reaching the maximum temperature of 50-65 ºC, doors and windows are opened to ventilate and lower the humidity.

(c) Aroma creating phase: The goals of this phase are to evaporate water and accumulate flavour compounds. During this 9-12 day incubation period, the temperature should not be lower than 45ºC, and the relative humidity should be 80%. After this phase, the temperature should decrease steadily to 30ºC.

Maturation: During maturation, the temperature of *Daqu* gradually equilibrates with the ambient temperature. Usually, maturation takes about 6 months.

2.4 Comparison of process conditions for different flavour *Daqu* types

Choice and formulation of ingredients

Moutai-Daqu, Luzhou-Daqu, Fen-Daqu, and *Xifeng-Daqu* are examples of four different flavours. Of these, both *Fen-Daqu* and *Xifeng-Daqu* (miscellaneous-flavour, low temperature) are made from barley and pea with the ratio of 6:4 or 7:3 (Shen, 2001b); while *Moutai-Daqu* and *Luzhou-Daqu* are produced from only pure wheat (Chen et al., 1995).

Stacking methods for Daqu

Light-flavour *Daqu* is a low-temperature *Daqu*. During the production of this type of *Daqu*, the stacking pattern should allow good ventilation and release of heat. Therefore all three stacking patterns of *Daqu* ("1" shape, "delta" shape and "fish bone" shape) are strictly applied according to the different incubation phases, and stacking is between 3 and 7 layers high (Wang et al., 2004). On the other hand, strong-flavour and sauce-flavour *Daqu* are high or medium temperature *Daqu*, so their production is geared towards maintaining high temperature and humidity levels. Consequently, stacking of the *Daqu* follows one pattern only, with 4-5 layers high throughout the incubation (Shen, 2001b). For miscellaneous-flavour *Daqu*, both stacking pattern and number of layers are diverse, because the ingredients, incubation and process differ as preferred by producing companies.

Table 2.2 Successive incubation periods for light-flavour *Daqu*

Incubation period	Explanation	Time range (d)	Temperature (°C)	Phenomena
Shangmei	Natural inoculation and activation of initial microbial growth	2-4	38	White spots of mycelium occur
Liangmei	Cooling down; to prevent damage from overheating; equilibration of moisture, microbial activity, and temperature	3-4	24-36	*Daqu* becomes harder
Chaohuo	Succession of dominate groups of microorganism	4-5	43-47	Most moisture is released
Dahuo	Enhanced proteolysis and accumulation of amino acids	7-8	43	Decrease of acidity and humidity
Houhuo	Pre-maturation*	4-5	34-38	Equilibrate temperature of *Daqu* to room temperature

Data compiled from (Shen, 2001b; Wang et al., 2004; Wang, 2000)
* This equilibration should take place prior to the "maturation" as shown in Figure 2.1.

Incubation of Daqu types

The production of light and miscellaneous-flavour *Daqu* follows distinct and specific temperature regimes, which are named the *Shangmei*, *Liangmei*, *Chaohuo*, *Dahuo* and *Houhuo* periods. These five stages are associated with the initiation of microbial growth, hardening, succession of dominant groups of microorganisms, enhancing proteolysis, and maturation. Table 2.2 gives detailed information for every period. *Shangmei,* also sometimes called *Shengyi* or *Guayi*, means "growth of filamentous fungal mycelium". Fungi occur as a natural inoculum on raw materials, water, rush mats, bran coat and environment. The mycelial development takes about 2-4 days. When white spots of mycelium occur on the surface of *Daqu*, the next period *Liangmei*, for cooling and hardening, is started. The key incubation period for microbial succession is "*Chaohuo*". As shown in Table 2.2, the temperature increases to about 43-47°C. During this incubation period, functional groups of microorganisms are favoured to grow into dominance, and cause the development of aroma compounds during *Dahuo* period. Finally, to allow the equilibration of moisture, acidy and enzyme activity, the *Houhuo* period is essential. As mentioned earlier, strong and sauce flavour *Daqu* require three stage incubation regimes. The scientific basis for these different scenarios is unclear and merits further investigation.

Sensory characteristics of Daqu

The different ingredients and incubation conditions will determine microbial succession and dominance, expressing distinct sensory characteristics such as colour, aroma and texture. Table 2.3 allows a comparison of physical and sensory characteristics of the four typical famous types of *Daqu*. Different *Daqu* have different sizes and weights. Sauce-flavour *Daqu* bricks are the biggest and heaviest (4.8 kg) whereas light-flavour *Daqu* bricks are the smallest and lightest (1.7 kg). *Daqu* types have their own specific texture and aroma. Because the incubation temperature of sauce-flavour *Daqu* is quite high, reaching up to 70°C, Maillard reactions result in colour gradients from brown inside to yellow on the surface. Under these hot incubation conditions, only a few microorganisms will be able to survive and dominate, and these are associated with the formation of a pungent flavour. On the other hand, the incubation temperature of light- and miscellaneous-flavour *Daqu* is

relatively lower (40-50°C), allowing for more fungal growth and the aroma is described as light and mushroom-like. Strong-flavour *Daqu* has a medium incubation temperature (50-60°C); at this range, thermophilic bacteria may become a dominant group, competing with other bacteria and moulds, and producing a strong flavour.

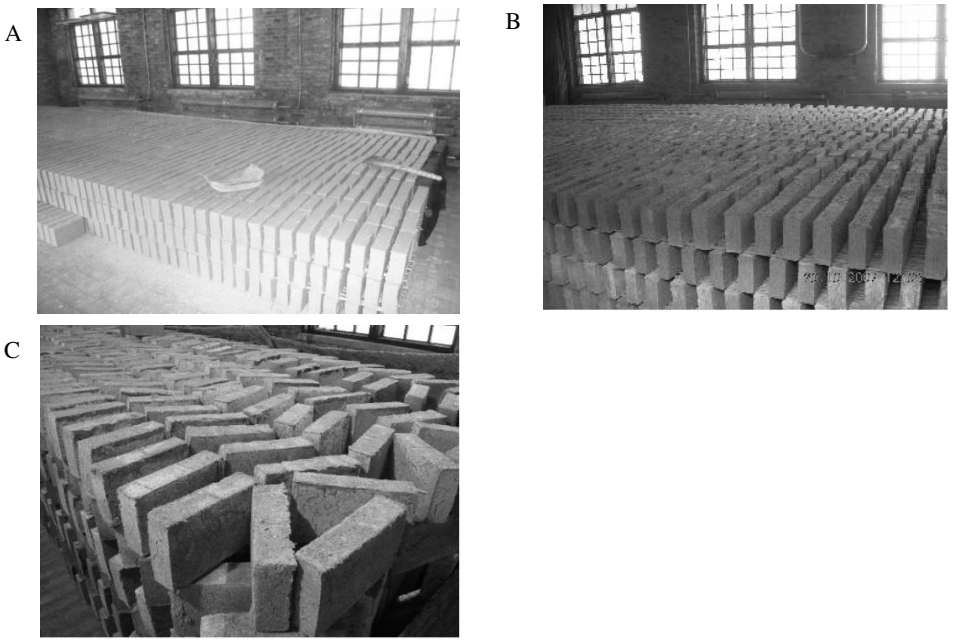

Figure 2.2 Stacking arrangements during incubation of *Daqu* (a) "1" shape; (b) "delta" shape; (c) "fish bone" shape.

2.5 Relationships between process conditions characteristics and functionality of *Daqu*

Daqu is produced from raw (uncooked) ingredients. Experience has shown that this results in better microbial and enzymatic activity for the production of liquor, because endogenous hydrolytic enzymes, such as β-glucanase (Xu et al., 2003) are present in barley. These enzymes can convert β-glucans to fermentable sugars and lead to reduced viscosity, which later can improve the exchange of substrates for second stage yeast alcoholic fermentation. Furthermore, some of the microorganisms grown in uncooked ingredients have been reported to produce acidic carboxyl proteases. This enzyme activity is important

in the degradation of denatured proteins and their conversion into amino acids, which in turn enhance microbial growth and the formation of trace levels of aroma compounds (Shen, 2001b).

2.6 Conclusion

In order to facilitate technological progress, improve control of quality, and reduce the variability in fermentation outcomes, qualitative and quantitative knowledge of the microbial diversity of *Daqu* is a prerequisite. Also required is a scientific understanding of the ecological role of processing conditions on microbial diversity and functionality.

As yet, limited work has been done to study these products with a view of upgrading traditional techniques. Therefore, the further study of microbial ecology during the production of *Daqu* and the ensuing alcoholic fermentation will be a considerable challenge. A better understanding of the eco-physiology of microorganisms of relevance for the specific types of Chinese liquors will stimulate the standardization and optimization of Chinese liquor production, allowing Chinese liquor to be increasingly recognized and appreciated by an international market.

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Table 2.3 Physical and sensory characteristics of major types and famous representatives of *Daqu*

Type of *Daqu*	**Representative *Daqu***	**Brick size[a]** (l/w/h; cm)	**Mass[a]** (kg / brick)	**Sensory properties**		
				Surface	**Texture**	**Aroma**
Sauce-flavour *Daqu*	*Moutai*	37 /23 /6.5	4.8 - 5.0	yellow	brown soft inside	pungent
Strong-flavour *Daqu*	*Luzhou*	34 /20 /5	2.5 - 2.8	white, white spots or microbial colonies visible	hard to cut	heavy
Light-flavour *Daqu*	*Fen*	27.5/16 /5.5	1.7 - 2.0	smooth, white, grain husks visible on edges	hard to cut, big particle size	light, mould flavour
Miscellaneous-flavour *Daqu*	*Xifeng Baiyunbian Site*	28 /18 /6	2.2 - 2.5	white, grain husks visible	hard to cut, small particle size	light, pleasant, mould flavour

[a] Approximate size and mass.

Chapter 3

Characterization of the microbial community in different types of *Daqu*

Abstract

Daqu is a fermentative saccharification agent that is used to initiate fermentation in the production of Chinese liquor and vinegar. Different types of *Daqu* can be distinguished based on the maximum fermentation temperature, location of production, and raw materials used. We aimed to characterize and distinguish the different types of *Daqu* using a culture-independent cloning method. The lowest microbial diversity was found in *Daqu* produced at high-temperature. Principal Component Analysis (PCA) was used to compare the bacterial composition of *Daqu* from different regions (i.e., northern *Daqu* and southern *Daqu*). *Staphylococcus gallinarum* and *Staphylococcus saprophyticus* were found in southern *Daqu*, and were absent in northern *Daqu*. The fungi *Saccharomycopsis fibuligera* and *Lichtheimia ramosa* dominated in low/medium-temperature *Daqu,* whereas *Thermomyces lanuginosus* occurred in high-temperature *Daqu*. Our study identified potential biomarkers for the different types of *Daqu,* which can be useful for quality control and technology development of liquor or vinegar production.

Zheng, X.W., Yan, Z., Nout, M.J.R., Boekhout, T., Han, B.Z., Zwietering, M.H., Smid, E.J., 2015. Characterization of the microbial community in different types of *Daqu* samples as revealed by 16S rRNA and 26S rRNA gene clone libraries. World Journal of Microbiology and Biotechnology 31, 199-208.

3.1 Introduction

Chinese liquor and vinegar have a long history of production and consumption and are produced through unique brewing processes. They are typically produced from cereals, such as sorghum, by solid-state fermentation using a natural fermentation starter termed *Daqu*. *Daqu* comprises a microbial community and is rich in enzymes. *Daqu* is made by a natural fermentation process running for a few weeks such that a microbial succession occurs (Zheng et al., 2011; Zheng et al., 2014). Being a major source of microorganisms and enzymes, *Daqu* is crucial for the quality, safety, and flavour of its derived products, such as liquor and vinegar. Different types of *Daqu* can be distinguished (Zheng et al., 2011), for instance, according to the maximum incubation temperature during the fermentation. *Daqu* can be grouped into three classes based on the production temperature: (i) high-temperature *Daqu* (60-70°C), (ii) medium-temperature *Daqu* (50-60°C), and (iii) low-temperature *Daqu* (40-50°C). According to the raw materials used for production, *Daqu* can be classified as single-grain *Daqu* or multi-grain *Daqu*. *Daqu* can also be classified geographically into southern and northern *Daqu*. Generally, southern *Daqu* is classified as a single-grain product produced at medium to high temperature. The northern variant is commonly a multi-grain, low to medium temperature *Daqu* (Shen, 2001a). Several studies have shown the diversity of the microbial community in *Daqu* (Wang et al., 2011b). We hypothesized that the microbial composition of *Daqu* correlates with environmental factors prevailing during the fermentation process. Thus, the microbial community in similarly classified *Daqu* is predicted to harbour common species or similar dominant groups of microorganisms.

The microbial community of *Daqu* has been analysed in previous studies using culture-dependent methods, such as isolation and enumeration on selective media (Li et al., 2009; Ma et al., 2011; Zheng et al., 2012), as well as by culture-independent methods, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and amplified fragment length polymorphism (Gao et al., 2010; Meng et al., 2010; Yan et al., 2012). In the present study, a semi-quantitative culture-independent cloning method was used for the analysis of microbial communities in *Daqu*. By comparing clone libraries, not only qualitative information on the composition of the microbial community is obtained,

but also quantitative information of the relative abundance of the identified species. The main objective of this study was to obtain an overview of the composition of the microbial communities in different types of *Daqu*. This analysis is expected to deliver potential biomarkers for fast and reliable verification of the authenticity of *Daqu* types.

3.2 Materials and Methods

Sampling

Eight types of brick-shaped *Daqu* were obtained from five commercial distilleries located in northern and south-western China. *Daqu* was produced and matured according to the procedures of the different distilleries. An overview of the types of *Daqu* and their technological parameters is presented in Table 3.1. In order to obtain adequate repetition, three blocks of each type of *Daqu* were randomly selected from each of the upper, middle, and lower stacked layers, and ground together. About 100 g of these *Daqu* powders was used as an experimental *Daqu* powder sample. Samples were then collected in sterile Stomacher® bags (Seward Laboratory Systems Inc., London, UK), transported to the laboratory in a cool box, and stored at -20°C until analysis.

DNA extraction and PCR amplification

DNA from eight powdered *Daqu* samples was extracted according to the method of Wang et al. (2008b) and diluted to a DNA concentration of 50 ng/µl. The 16S rRNA bacterial gene was amplified using universal primers "B-for" (5′-AGAGTTTGATCCTGGCTCAG-3′) and "B-rev" (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al., 1989). The D1/D2 domain of the 26S rRNA fungal gene was amplified using universal primer "NL1" (5′-TGCTGGAGCCATGGATC-3′) and reverse primer "RLR3R" (5′-GGTCCGTGTTTCAAGAC-3′) (Okoli et al., 2007). PCR was performed in a total reaction volume of 50 µl containing 26.6 µl ddH_2O, 5 µl PCR buffer, 3 µl $MgCl_2$ (25 mM), 10 µl dNTP (2 mM), 2 µl of each primer (10 µM), 1 µl DNA template (approximately 50 ng), and 0.4 µl Taq DNA polymerase (5 U/µl) (Fermentas, USA). PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, USA) with the following PCR conditions: initial denaturation for 5 min at 94°C; 35 cycles each consisting of 30 s at 94°C,

20 s at 56°C (bacteria) or 52°C (fungi), and 1 min at 72°C; and extension of incomplete products for 7 min at 72°C, followed by cooling at 4°C. The sizes and quantities of the PCR products were determined using 1.5% (wt/vol) agarose gel electrophoresis. The PCR products were analysed by electrophoresis and then stored at -20°C for future experiments.

DNA clone library construction

Clone libraries of 16S rDNA and 26S rDNA amplicons from *Daqu* samples were constructed. Amplicons derived from PCR products were purified with a QIAquick PCR purification Kit (Qiagen, Hilden, Germany), cloned using a pGEM-T Easy Cloning Kit (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 High Efficiency Competent Cells (Promega), following the manufacturer's instructions. Around 90 positive clones (white colonies) were randomly picked from the plates of each sample. These plasmid-harbouring clones were transferred with a sterile toothpick into 50 µl of Tris-EDTA buffer, lysed, and amplified with "T7" and "Sp6 pGem-T"-specific primers to confirm the appropriate size of the insert (approximately 1,500 bp for bacteria and 700 bp for fungi).

Table 3.1 *Daqu* samples investigated

Sample code	Type	Raw materials	Maximum temperature reached during the fermentation (°C)	Location
9-H-S-W*	High-temperature	wheat	62.7±0.2	28.32° N (S)
5-H-S-W	High-temperature	wheat	65.2±0.1	28.88° N (S)
8-M-N-BP	Medium- temperature	barley and pea	54.2±0.1	40.02° N (N)
5-M-S-W	Medium- temperature	wheat	54.6±0.1	28.88° N (S)
4-M-S-W	Medium- temperature	wheat	56.5±0.2	28.55° N (S)
7-L-N-BP'	Low-temperature	barley and pea	50.1±0.2	37.31° N (N)
7-L-N-BP	Low-temperature	barley and pea	45.2±0.1	37.31° N (N)
8-L-N-BP	Low-temperature	barley and pea	50.7±0.1	40.02° N (N)

*indicate the codes of *Daqu* samples: The number in codes means the name of liquor factory; the letter "H" "M" and "L" indicates the high temperature *Daqu*, Medium temperature *Daqu* and Low temperature *Daqu*, respectively. The letter "N" and "S" indicates that the *Daqu* is produced in northern China and southern China, respectively. The letter "W" and "BP" indicates that the *Daqu* is produced from wheat and "barley and peas", respectively. The only difference between samples 7-L-N-BP and 7-L-N-BP' is maximum temperature.

Clones containing the plasmid with an insert were sent for sequencing at the Beijing Genomics Institute (Beijing, China). Sequences were assembled and edited with Seqman II software (DNAStar Inc., Madison, WI, USA) and aligned with Megalign (DNAStar Inc.). Chimeric sequences in the clone library were identified and discarded using the software package Chromas v.2.31 (Technelysium Pty Ltd.). The nucleotide sequences obtained were identified in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/) to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

Calculation of species diversity indices

To determine the diversity of species in *Daqu* samples (as revealed by cloning), Shannon's diversity index ($H' = -\sum p_i \ln(p_i)$, where p_i is the proportion of taxon i), was calculated.

Statistical analysis

The composition of microbiological communities in all *Daqu* samples was analysed by PCA using the software package SIMCA-P 12.0 (Umetrics, Umea, Sweden) to cluster the samples into different groups. Samples were plotted in two dimensions based on scores for the first two principal components to evaluate relationships among samples. The proportion of variance explained by each principal component was calculated.

3.3 Results

Composition of microbial communities in Daqu

The composition of the microbial communities in *Daqu*, representing three temperature types obtained from five factories, is shown in Table 3.2. About 69 bacterial species and 19 fungal species were detected by the cloning method. Only three species (*Bacillus licheniformis*, *Saccharomycoposis fibuligera* and one uncultured bacterium) were detected in all types of *Daqu*. Twenty bacterial species and 11 fungal species were found in high-temperature *Daqu*, but only five species, i.e. *B. licheniformis*, *Enterobacter* sp., *Pichia kudriavzevii*, *Sm. fibuligera*, and *Thermomyces lanuginosus* were found in all high-temperature *Daqu* samples. Forty-three bacterial species and 10 fungal species were found

in medium-temperature *Daqu*, but only four species, i.e. *B. licheniformis*, *Bacillus* sp., *Sm. fibuligera*, and *Lichtheimia ramosa* were found in all medium-temperature *Daqu*. Twenty-nine bacterial species and four fungal species were found in low-temperature *Daqu*, with *B. licheniformis*, *Sm. fibuligera*, *Lichtheimia corymbifera*, and *P. kudriavzevii* as common species occurring in all low-temperature *Daqu*. The lowest bacterial diversity ($H^{'}$ = 1.19) was found in sample 9-H-S-W, and the highest bacterial diversity ($H^{'}$ = 3.40) was found in sample 4-M-S-W. For fungi, the lowest diversity ($H^{'}$ = 0.49) was in sample 8-M-N-BP, and the highest ($H^{'}$ = 1.59) in sample 5-H-S-W (Table 3.2).

Characteristics of different types of Daqu

Group-wise PCA comparisons of the bacterial and fungal composition of the different types of *Daqu* were constructed (Figures 3.1a and 3.2a). The loading plots indicate the species that are responsible for the separation of the clusters (Figure 3.1b and Figure 3.2b).

Based on bacteria detected, five of the eight samples (8-L-N-BP, 4-M-S-W, 5-H-S-W, 5-M-S-W and 9-H-S-W) clustered together (cluster 1). Furthermore, samples 7-L-N-BP and 7-L-N-BP$^{'}$ clustered together (cluster 2) and sample 8-M-N-BP was separated from all other samples (Figure 3.1a). With one exception (i.e., sample 8-L-N-BP), all *Daqu* samples in the main cluster 1 are from southern China, while the *Daqu* samples 7-L-N-BP, 7-L-N-BP' and 8-M-N-BP are from northern China. The loading plot (Figure 3.1b) indicates the bacterial species that contributed to this discrimination. The microbial species that most significantly characterized different types by their increased relative abundance were in cluster 1 are: *Saccharypolyspora rosea*, *Streptomyces albus*, *Thermomonospora chromogena*, *Staphylococcus gallinarum*, *Staphylococus* sp., *Staphylococcus saprophyticus*, *Bacillus* sp., *Enterobacter cowanii*, and *Escherichia hermannii*. In cluster 2, *Weissella confusa* showed a marked increase in relative abundance and in cluster 3 the species *Thermoactinomyces sanguinis*, *Saccharopolyspora* sp., *Saccharopolyspora rectivirgula* were detected with an increased abundance in the microbial population.

The PCA of three temperature types of *Daqu* based on fungal composition (see Figure 3.2), showed three groups (Figure 3.2a). The species that most significantly characterized the different clusters by their fungal composition were in cluster 1 *Rhizomucor pusillus*,

Absidia idahoenis and *L. corymbifera*; in cluster 2 *Sm. fibuligera* and *L. ramosa* and in cluster 3 *T. lanuginosus* and *Aspergillus flavus* (Figure 3.2b).

3.4 Discussion

Knowledge of the microbiota of *Daqu* is still far from complete. Therefore, this study was initiated to understand the composition of the microbial community in three temperature types of this saccharafication agent. *Daqu* is made from different ingredients (barley, pea, or wheat), and is produced in different locations in China, each location applying different fermentation conditions. The most variable parameter is the maximum temperature of fermentation. Three arbitrary classes can be distinguished with regard to the latter parameter: (i) high-temperature, (ii) medium-temperature, and (iii) low-temperature processes for *Daqu* production. It is expected that the relative abundance of several of the identified microorganisms correlates with specific environmental conditions. For instance, the prevailing temperature is expected to have a major selective effect on the microbiota.

The presence or relative abundance of other microorganisms could be associated with available substrates for fermentation, the location of production facility, and unique factory conditions. The lowest bacterial diversity as measured by the Shannon index (H'=1.19) was found in a high-temperature *Daqu* (9-H-S-W). Temperatures higher than 65°C occurred during the production of high-temperature *Daqu*, and such temperatures only permit the survival and growth of thermophilic or thermotolerant bacteria and fungi, such as *Bacillus* spp. and *Thermomyces* spp., respectively (Moretti et al., 2012). This explanation is in line with our observations of the high abundance of *B. licheniformis*, and *T. lanuginosus* in high-temperature *Daqu* (samples 9-H-S-W and 5-H-S-W). Samples 5-H-S-W and 5-M-S-W were made from the same raw materials (wheat) and produced in the same factory; they only varied in their fermentation temperatures (about 10°C differences). The comparison of the microbial diversity between samples 5-H-S-W and 5-M-S-W revealed a reduction in fungal diversity upon elevation of the fermentation temperature: sample 5-H-S-W had a lower number of species and a lower value of Shannon's diversity index compared to sample 5-M-S-W. Two samples obtained from factory 8 (i.e., 8-L-N-BP and 8-M-N-BP) were also produced in the same factory and they reavealed the same trend: the higher the

Table 3.2 Microbial composition of three types of *Daqu*

Microorganisms	High-temperature *Daqu* (60-70°C)		Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)		
	9-H-S-W	*5-H-S-W*	*8-M-N-BP*	*5-M-S-W*	*4-M-S-W*	*7-L-N-BP*	*7-L-N-BP'*	*8-L-N-BP*
Bacteria								
Acinetobacter baumannii					[7]			
Acinetobacter sp.					[1]			
Actinopolyspora salina			[1]					
Altererythrobacter sp.					[1]			
Bacillus atrophaeus						[1]		
Bacillus licheniformis	[27]	[21]	[5]	[18]	[4]	[4]	[10]	[55]
Bacillus oleronius		[1]			[1]			
Bacillus pumilus						[1]	[3]	
Bacillus sp.		[29]		[21]		[1]	[2]	[6]
Bacillus subtilis					[3]		[8]	
Bacillus shackletonii								[1]
Bacillus sonorensis						[3]		
Brevundimonas sp.					[1]			
Corynebacterium sp.						[1]		
Desmospora activa			[1]					
Endophytic bacterium					[4]			
Enterobacter asburiae		[2]			[7]			
Enterobacter cowanii	[21]							

Table 3.2 Microbial composition of three types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)		Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)		
	9-H-S-W	*5-H-S-W*	*8-M-N-BP*	*5-M-S-W*	*4-M-S-W*	*7-L-N-BP*	*7-L-N-BP'*	*8-L-N-BP*
Bacteria								
Enterobacter cloacae		[1]						
Enterobacter sp.	[4]	[5]			[11]		[5]	[1]
Enterococcus canintestini						[1]		
Enterococcus saccharolyticus						[1]		
Escherichia hermannii	[28]							
Kocuria sp.					[1]			
Klebsiella pneumonia					[8]			
Klebsiella sp.					[2]			
Klebsiella variicola					[5]			
Kurthia gibsonii						[1]		
Lactobacillus farciminis						[2]	[4]	
Lactobacillus fermentum					[1]			
Leclercia sp.					[1]			
Legionella taurinensis					[1]			
Leuconostoc citreum					[1]			
Leuconostoc pseudomesenteroides						[1]		
Microbispora bispora			[2]					
Myxococcus xanthus		[1]						

Table 3.2 Microbial composition of three types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)		Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)		
	9-H-S-W	*5-H-S-W*	*8-M-N-BP*	*5-M-S-W*	*4-M-S-W*	*7-L-N-BP*	*7-L-N-BP'*	*8-L-N-BP*
Bacteria			[3]					[11]
Oceanobacillus sp.								
Pantoea agglomerans					[1]	[1]		
Pantoea sp.		[2]						
Pantoea vagans						[2]		
Patulibacter minatonensis					[1]		[4]	
Pediococcus acidilactici			[2]					
Nilaparvata lugens					[1]			
Saccharopolyspora hordei		[2]	[2]		[1]			
Saccharopolyspora rectivirgula			[13]					[4]
Saccharopolyspora rosea			[6]		[2]			
Saccharopolyspora sp.			[12]					[3]
Saccharopolyspora spinosa			[1]					
Sphingomonas aurantiaca						[1]	[2]	
Staphylococcus gallinarum	[2]	[2]		[9]	[1]			
Staphylococcus saprophyticus	[2]	[3]		[20]	[3]			

Table 3.2 Microbial composition of three types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)		Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)		
	9-H-S-W	*5-H-S-W*	*8-M-N-BP*	*5-M-S-W*	*4-M-S-W*	*7-L-N-BP*	*7-L-N-BP'*	*8-L-N-BP*
Bacteria								
Staphylococcus sciuri						[4]	[3]	
Staphylococcus sp.				[18]	[5]	[6]	[7]	
Stenotrophomonas maltophilia			[1]		[6]			
Streptomyces cacaoi								[3]
Streptomyces albus			[4]					[3]
Streptomyces sp.			[1]					
Tepidanaerobacter sp.		[2]						
Thermoactinomycetaceae bacterium		[2]						
Thermoactinomyces sanguinis		[1]	[36]		[1]			[6]
Thermoactinomyces vulgaris		[1]						
Thermobispora bispora			[2]					
Thermomonospora chromogena			[3]					
Weissella cibaria		[1]				[5]	[12]	
Weissella confusa		[2]			[10]	[50]	[38]	

Table 3.2 Microbial composition of three types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* (60-70°C)		Medium-temperature *Daqu* (50-60°C)			Low-temperature *Daqu* (40-50°C)		
	9-H-S-W	*5-H-S-W*	*8-M-N-BP*	*5-M-S-W*	*4-M-S-W*	*7-L-N-BP*	*7-L-N-BP'*	*8-L-N-BP*
Bacteria								
Weissella paramesenteroides						[2]		
Uncultured bacterium	[3]	[9]	[1]	[1]	[3]	[2]	[1]	[3]
Fungi								
Absidia idahoenis					[20]			
Aspergillus flavus	[3]							
Aspergillus fumigatus	[2]			[2]				
Coelometopinae sp		[1]						
Cucujus clavipes		[5]						
Eurotium amstelodami				[2]				
Lichtheimia ramosa			[5]	[21]	[5]			
Lichtheimia corymbifera		[1]			[15]	[11]	[18]	[15]
Pichia kudriavzevii	[31]	[21]				[21]	[19]	[21]
Pselaphacus signatus		[1]						
Pselaphacus vitticollis		[5]						
Rhizomucor miehei					[2]			
Rhizomucor pusillus					[39]			

Table 3.2 Microbial composition of three types of *Daqu* (continued)

Microorganisms	High-temperature *Daqu* （60-70°C）		Medium-temperature *Daqu* （50-60°C）			Low-temperature *Daqu* （40-50°C）		
	9-H-S-W	*5-H-S-W*	*8-M-N-BP*	*5-M-S-W*	*4-M-S-W*	*7-L-N-BP*	*7-L-N-BP'*	*8-L-N-BP*
Fungi								
Saccharomycopsis fibuligera	[21]	[20]	[52]	[42]	[7]	[53]	[48]	[45]
Stephanoascus ciferrii					[1]			
Talaromyces luteus		[1]						
Thermomyces lanuginosus	[28]	[30]						
Trichomonascus ciferrii				[2]				
Wickerhamomyces anomalus						[12]		
Total	[172]	[167]	[153]	[156]	[184]	[187]	[184]	[177]
Bacterial diversity scores (Shannon's diversity, H_b')	1.19	2.03	2.13	1.62	3.40	1.92	1.88	1.61
Fungal diversity scores (Shannon's diversity, H_f')	1.28	0.97	0.49	1.59	1.49	1.16	1.08	0.98

The number in the brackets [ ] indicates the number of clones isolated.

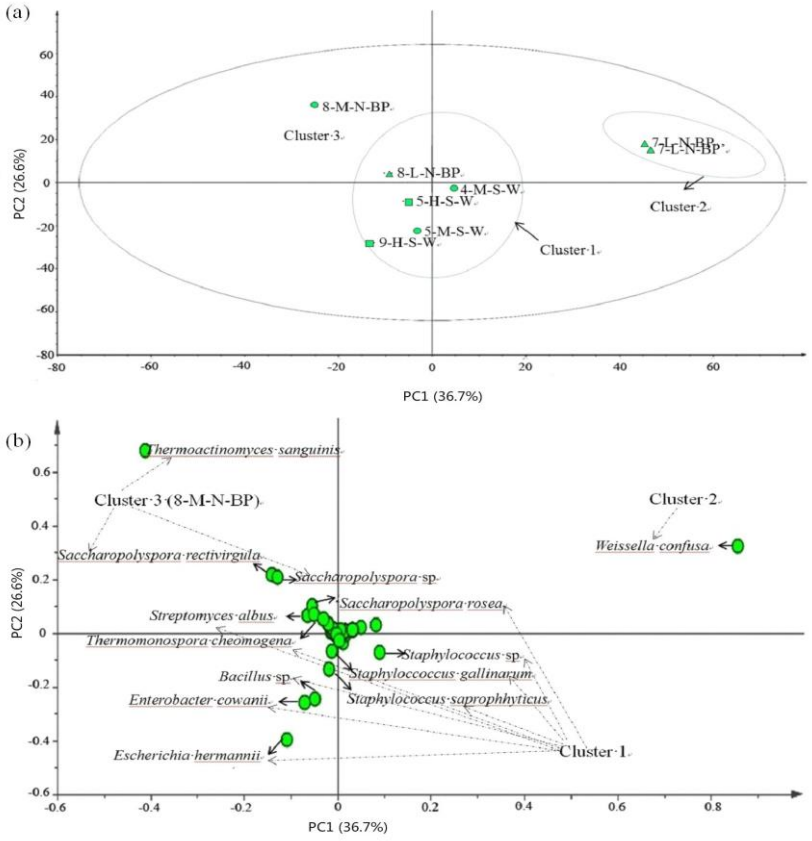

Figure 3.1 PCA of *Daqu* extracts on bacterial composition (a) Score plots of three temperature types of *Daqu* (b) Loading plots of PC1 and PC2. ▲low-temperature *Daqu*; ● medium-temperature *Daqu*; ■ high-temperature *Daqu*.

temperature, the lower the diversity in fungal composition (Table 3.2). However, the bacterial composition revealed an opposite trend. This indicates that the bacterial composition, in comparison to the fungal composition, is affected more by other factors such as moisture content and oxygen condition. The production technique used could be another factor affecting the bacterial community in *Daqu.* One specific technique called "back-slopping" was used in factory 9. The *Daqu* (4-8%) that was produced one year ago (named "mother *Daqu*") was added to the raw materials, and the mixture was used to carry out the *Daqu* fermentation. On the one hand, the mild acidity of the "mother *Daqu*" could

inhibit the growth of fungi (Li, 2013), on the other hand however, the dominant microorganisms in the "mother *Daqu*" could dominate the *Daqu* fermentation, thereby suppressing the less prevalent microorganisms. This presumably explains why only seven bacterial species were detected in the sample 9-H-S-W.

B.licheniformis, *Sm. fibuligera* and one uncultured bacterium were detected in all tested *Daqu* samples. This result is in agreement with the study of Wang et al. (2011b). *B. licheniformis* is a ubiquitous spore-forming bacterium associated with a variety of fermented food products (Lima et al., 2012; Ramos et al., 2010; Wang et al., 2011.), and it is a well-known producer of proteases and amylases (Karataş et al., 2013). The high relative abundance of *B. licheniformis* in *Daqu* suggests that it plays an important role in flavour formation in products such as Chinese liquor and vinegar by hydrolysis of complex carbohydrates and proteins during fermentation. *B. licheniformis* was found to produce more than 70 metabolites, most of which are flavour compounds and flavour precursors important for the aroma of fermented products (Yan et al., 2007). Yan et al. (2013b) reported high levels of acetic acid and lactic acid produced by *B. licheniformis*. These organic acids may give rise to a variety of aroma compounds by esterification with ethanol. This corresponds well with the fact that the key aroma compounds in light-flavour liquor, such as *Fen*-liquor (factory 7), are mainly ethyl acetate and ethyl lactate. An abundance of *B. licheniformis*, *Bacillus subtilis*, and non-specified *Bacillus* sp. was found in high-temperature *Daqu*, as has been observed elsewhere (Huang et al., 2006; Yan et al., 2007). Wu et al. (2009a) analysed the metabolite composition in different types of *Daqu*, and found higher concentrations of amino acids, such as isoleucine and leucine, in high-temperature *Daqu.* This correlates with the abundance of *Bacillus* spp. in high-temperature *Daqu*, since *Bacillus* spp. were shown to be important thermophilic protease producers (Zhang et al., 2007b).

Sm. fibuligera was encountered in different types of *Daqu* (Wang et al., 2011b). The role of *Sm. fibuligera* in *Daqu* production may be the secretion of amylases, acid proteases, and β-glucosidases, which have high potential application in the fermentation industry (Chi et al., 2009). *Sm. fibuligera* also has been reported to degrade and assimilate raw starch as a

carbon source (Chi et al., 2009); thus, it may contribute to the formation of fermentable carbohydrates for subsequent alcoholic fermentation.

In the current study, various genera of lactic acid bacteria (LAB) were identified in *Daqu* samples, including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weisella*. In general, LAB was found in low abundance, except *Ws. confusa* which was found at high abundance in two *Daqu* samples (i.e., 7-L-N-BP and 7-L-N-BP'). Based on this, the high abundance of *Ws. confusa* can be used potentially to distinguish *Daqu* from factory 7 from *Daqu* samples originating from other production locations. Among the LAB species, *Lactobacillus fermentum*, *Leuconostoc citreum*, *Pediococcus acidilactici*, *Ws. confusa*, and *Ws. cibaria* were reported in earlier studies on other types of *Daqu* (Wang et al., 2011b; Zheng et al., 2012). Several studies mentioned the importance of LAB during the production of *Daqu*, but it was only found at high abundance during the beginning of the *Daqu* production process (Lei, 2011). The study of Katina et al. (2002) indicated that some species of *Lactobacillus* inhibit the growth of *Bacillus* spp., especially *B. subtilis* and *B. licheniformis*. This might explain that a high abundance of LAB was present at early stages of *Daqu* production. However, the increase in temperature throughout the fermentation process results in the fast growth of thermophilic bacteria such as *Bacillus* spp. in *Daqu* and slower growth of mesophilic LAB (Lei, 2011). This might explain the low abundance of LAB in the final *Daqu* products.

Thirteen species of actinomycetes were detected in *Daqu*, i.e., *Actinopolyspora salina*, *Saccharopolyspora hordei*, *Sac. rectivirgula*, *Sac. rosea*, *Saccharopolyspora spinosa*, *Saccharopolyspora* sp., *Streptomyces cacaoi*, *Str. albus*, *Streptomyces* sp., *T. sanguinis*, *T. chromogena*, *Thermoactinomyces* bacterium and *Thermobispora bispora* (Table 3.2). Of these, *Sac. rectivirgula*, *Saccharopolyspora* sp. and *Str. albus* were detected particularly in 8-M-N-BP and 8-L-N-BP, which both originate from the same factory (i.e., factory 8), but were processed at different fermentation temperatures. Since *Sac. rectivirgula*, *Saccharopolyspora* sp. and *Str. albus* were not present in other *Daqu* samples; these three actinomycetes may represent the "house microbiota" of the factory 8. Wang et al. (2012a) monitored the presence of actinomycetes during the production of liquor and observed that >80% of all the identified actinomycetes (especially *Streptomyces* spp.) originated from the

air in the production room. This result was in line with our hypothesis that *Sac. rectivirgula*, *Saccharopolyspora* sp., and *Str. albus* belong to the "house microbiota". To date, no studies have been published on the role of actinomycetes in the production of *Daqu*, even though they commonly occur in *Daqu*. However, other studies have reported the ability of *Thermoactinomycetes* sp. and *Streptomyces* spp. to secrete alkaline phosphatase, esterase, lipid esterase, and phosphate hydrolase (Liu et al., 2012b; Wang et al., 2012a), which might play important roles in the formation of the flavour compounds or flavour precursors during *Daqu* fermentation processes.

Analysis of different samples of southern *Daqu* revealed that *Staphylococcus* spp., especially *St. gallinarum* and *St. saprophyticus* could be considered as biomarkers of southern *Daqu* (Gao et al., 2010; Wang et al., 2012b), since these two bacteria were absent in the northern *Daqu* samples (8-M-N-BP, 8-L-N-BP, 7-L-N-BP and 7-L-N-BP'). This indicates that the bacterial community of *Daqu* is highly dependent on locations. In addition, the selection of raw materials and the environmental conditions (soil, air, etc.) could also influence the bacterial community in *Daqu* (Gao, 2010; Xu et al., 2004). *Klebsiella* was found to be heavily associated with the soil used for planting wheat. Only one *Daqu* sample (4-M-S-W) contained *Klebsiella* (including *Klebsiella pneumonia*, *Klebsiella variicola*, and *Klebsiella* sp.), and its presence probably indicates soil contamination. Another study reports that bacteria belonging to the genera *Bacillus* and *Micrococcus* were the only dominant bacterial species in wheat (Xu et al., 2004). The high number of *Bacillus* sp. in the *Daqu* samples, 5-H-S-W and 5-M-S-W, may be attributed to the dominance of *Bacillus* in the wheat samples originating from factory 5.

Temperature is an important environmental parameter that affects the growth and survival of microorganisms and, consequently, largely contributes to the microbial community structure of *Daqu* (Wang et al., 2011b). In general, yeasts and moulds are more sensitive to heat than bacteria (Wang et al., 2011b). PCA confirmed grouping of the composition of the fungal communities of *Daqu* according to the fermentation temperature (Figure 3.2). *T. lanuginosus*, a thermophilic fungus that survives at temperatures higher than 60°C (Singh et al., 2003), is a candidate biomarker for high-temperature *Daqu*. *T. lanuginosus* has been reported to be an efficient xylanase producer, and the xylanase from

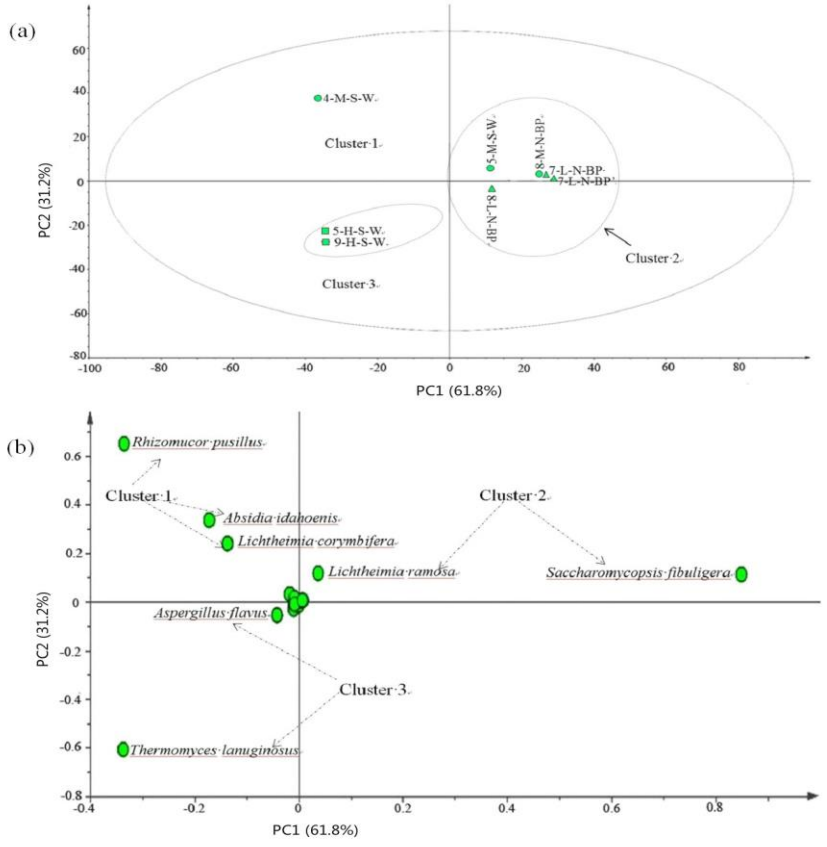

Figure 3.2 PCA of *Daqu* extracts on fungal composition (a) Score plots of three temperature types of *Daqu* (b) Loading plots of PC1 and PC2. ▲low-temperature *Daqu*; ● medium-temperature *Daqu*; ■ high-temperature *Daqu*.

this fungus is active over a wide pH range (Singh et al., 2003). This might imply that *T. lanuginosus* also plays a role in degrading xylan, as reported previously (Archana et al., 1997). *Sm. fibuligera* occurred in all tested types of *Daqu* and represented about 50% of the total fungal community in low/medium-temperature *Daqu*. This observation suggests an important role for this species in *Daqu*. *L. ramosa* is known to occur on wheat (Shang et al., 2012). Liu et al. (2010b) compared the microbial diversity on wheat and its derived *Daqu*, and observed that *L. ramosa* and *R. pusillus* occurred in both *Daqu* and wheat. In our study, *R. pusillus* was present in relatively high numbers in the *Daqu* sample 4-M-S-W, and this species probably originated from the wheat used in factory 4 (Xu et al., 2004). A

comparison of dominant microorganisms in different wheat varieties (Xu et al., 2004) revealed two dominating fungal genera (*Rhizopus* and *Aspergillus*) in wheat. Therefore, the *Asp. flavus* and *Aspergillus fumigatus* species detected in the southern *Daqu* samples (9-H-S-W and 5-M-S-W) could possibly be associated with the wheat used. All these findings indicate that the fungal communities in *Daqu* also depend on the raw materials formulation used during production.

Sample 9-H-S-W showed a relatively high abundance of Enterobacteriaceae (*Ent. cowanii* and *E. hermannii*) and this may indicate problems with hygienic processing in factory 9. Also, 9-H-S-W was the only sample with *Aspergillus flavus* (but at low abundance). Although we did not study the effect of *Asp. flavus* on the quality and safety of *Daqu*, the fact that this species is potentially able to produce aflatoxins, indicates that factory 9 requires a more strict quality control than other factories during the whole *Daqu* production process. Fortunately, this fungus was not observed in other types of *Daqu*, and therefore we do not regard this as a potential safety risk during *Daqu* production in general.

Until now, little attempt has been made to compare the microbial community structures of different types of *Daqu*. We have demonstrated that the fungal diversity in *Daqu* is highly influenced by fermentation temperature and raw materials, and that the bacterial diversity is influenced by fermentation temperature and geographic environment (i.e. climate, water, and air). The microbial communities of different types of *Daqu* samples differed significantly from each other. However, the relative abundances of species belonging to the genus *Bacillus* were higher than that of species of other bacterial genera. Among the *Bacillus* species, *B. licheniformis* was predominant and found in all *Daqu* samples, consistent with previous studies (Yao et al., 2005b; Zheng et al., 2011). On the other hand, each type of *Daqu* contained a high proportion of sample-specific bacteria. These bacteria and fungi are regarded as candidate biomarkers to distinguish different types of *Daqu*.

Differences in abundance of specific microorganisms present in *Daqu* samples as a function of regional origin potentially facilitate the selection of starters for creation of unique, region-specific flavours. Further research is required to establish the impact of *Daqu* composition on other quality aspects such as health effect of its derived product. This

work may help liquor and vinegar industries to understand the microbial ecology of *Daqu*, and this enables further optimization of using different types of *Daqu* for liquor and vinegar production.

Acknowledgements

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Chapter 4

Distribution of microbiota in a Chinese fermentation starter (*Fen-Daqu*) by culture-dependent and culture-independent methods - comparison of inner and outer *Daqu*

Abstract

Daqu is a traditional fermentation starter that is used for Chinese liquor production. Although partly mechanized, its manufacturing process has remained traditional. We investigated the microbial diversity of *Fen-Daqu*, a starter for light-flavour liquor, using combined culture-dependent and culture-independent approaches (PCR-DGGE). A total of 190 microbial strains, comprising 109 bacteria and 81 yeasts and moulds, were isolated and identified on the basis of the sequences of their 16S rDNA (bacteria) and 26S rDNA and ITS regions (fungi). DGGE of DNA extracted from *Daqu* was used to complement the culture-dependent method in order to include non-cultivable microbes. Both approaches revealed that *Bacillus licheniformis* was an abundant bacterial species, and *Saccharomycopsis fibuligera*, *Wickerhamomyces anomalus*, and *Pichia kudriavzevii* were the most common yeasts encountered in *Fen-Daqu*. Six genera of moulds (*Absidia*, *Aspergillus*, *Mucor*, *Rhizopus*, *Rhizomucor* and *Penicillium*) were found. The potential function of these microorganisms in starters for alcoholic fermentation is discussed. In general the culture-based findings overlapped with those obtained by DGGE by a large extent. However, *Weissella cibaria*, *Weissella confusa*, *Staphylococcus saprophyticus*, *Enterobacter aerogenes*, *Lactobacillus sanfranciscensis*, *Lactobacillus lactis*, and *Bacillus megaterium* were only revealed by DGGE.

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 Microbiology 31, 293-300.

4.1 Introduction

Chinese liquor is one of the world's oldest distilled alcoholic beverages, and plays an important role in Chinese culture and people's daily life. The total annual production of Chinese liquor was estimated at approximately 5 million metric tons per year (Anonymous, 2008). The popularity of Chinese liquor is in part due to the diversity of flavours. Chinese liquor flavours can be distinguished as strong-flavour [Nongxiang in Chinese](Zhang et al., 2012), light-flavour [Qingxiang in Chinese] and sauce-flavour [Jiangxiang in Chinese] (Shen, 2001a; Wu et al., 2009a; Zhang et al., 2011a). The liquor is made from cereals, mainly sorghum, by fermentation, distillation and maturation. Mixed culture starters (Fan et al., 2007), which are locally called "*Daqu*", are used as inoculum for the solid-state fermentation, as shown in Figure 4.1. The cooked cereals are inoculated by mixing with approximately 9-10% powdered *Daqu* and this mixture is fermented in sealed jars for 21-28 days at 10-16°C. *Daqu* is an important saccharifying and fermenting agent for the production of Chinese liquor and traditional vinegar, and has a significant impact on the flavour of the final product (Shen, 2001a; Zheng et al., 2011).

Daqu itself is also made by fermentation, which takes about one month. This is followed a storage period of 6 months for maturation. The manufacture of *Daqu* by traditional uncontrolled solid-state fermentation techniques results in products with inconsistent quality; this causes large losses of unsuccessful products which go to waste as animal feed (Shen et al., 2005). Therefore, standardization of the *Daqu* production process is an important objective for Chinese liquor producers. So-far, this has resulted in the application of modernized machinery, but little attention has been given so-far to the functional composition, i.e. the microbiota of *Daqu,* in view of controlling its fermentation.

Fen-Daqu is a light-flavour *Daqu* from Shanxi province, which is made from barley and pea, and contains around 50% of starch (Zheng et al., 2011). "Fen-type" liquor has a flavour that is described as pure and sweet, with a refreshing aftertaste (Xiong, 2005a). During the production of *Daqu* by solid-state fermentation, the temperature in the *Daqu* blocks may increase up to 60°C as a result of metabolic heat. In *Fen-Daqu* production, care is taken to restrict maximum temperatures to <50°C by ventilation. Most microorganisms

are expected to grow and survive these temperatures, and, therefore, *Fen-Daqu* is expected to contain a diverse microbial community.

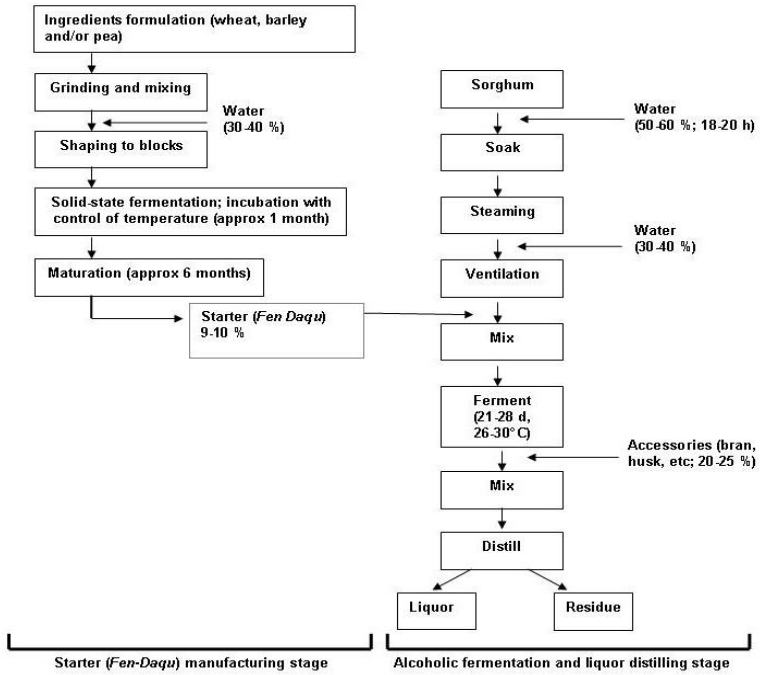

Figure 4.1 Process diagram for the production of *Fen-Daqu* and its role in the production process of Chinese liquor.

Traditional microbiological methods such as enumeration using different general and selective media can provide (i) quantitative data on the occurrence of different groups of microorganisms and (ii) isolated pure cultures for experimental fermentations. However, this classical culture-dependent approach only reveals the cultivable microbes. Therefore, molecular microbiological methods such as analysis of direct DNA extracts by PCR-T/DGGE, pyrosequencing, sequencing of clone libraries, etc. are useful additions to realize rapid and high throughput microbial communities analysis (Aquilanti et al., 2007; Camu et al., 2007; Iacumin et al., 2009; Papalexandratou et al., 2011a; Papalexandratou et al., 2011b; Thanh et al., 2008). In this study we report an investigation of the microbial community in *Fen-Daqu* using combined culture-dependent and -independent approaches.

4.2 Materials and Methods

Sample collection and storage of Daqu

Fen-Daqu was obtained from Shanxi Xinghuacun Fenjiu Group Company, the largest producer of light-flavour liquor. The dimensions of *Daqu* blocks are approximately 27 × 16 × 6 cm, weighing around 1.7 kg each. *Fen-Daqu* is fermented and matured in stacked layers of blocks. Three *Daqu* blocks (having been stored for 6 months of maturation) were randomly selected from upper, middle and lower layers in order to obtain an adequate representation. Before performing microbiological analysis, each *Daqu* block was separated into two parts, namely its outer greyish-coloured surface layer of 1 cm thick, and the remaining brownish-coloured inner part. Both inner and outer parts were weighed before being ground to powder in an alcohol-disinfected grinder (Krups 75, model F203); the powder was kept in plastic jars at 4°C during the experimental work.

Microbiological analysis

Samples of *Daqu* powder (10 gram each) were transferred to stomacher bags and homogenized with 90 ml sterile PPS (Peptone Physiological Salt) solution containing (g/l) peptone (Oxoid LP0034) 1, and NaCl 8.5, in a Stomacher Lab-blender 400, twice 1 minute at high speed. Appropriate serial dilutions were prepared using the same diluent. Unless otherwise indicated, 1 ml portions of the diluted suspension were mixed with molten (45°C) medium and poured in duplicate counting plates of the media described below.

Total aerobic and anaerobic bacteria were enumerated on Plate Count Agar (PCA; Oxoid CM035) and Reinforced Clostridial Agar (RCA; Oxoid CM0151), respectively. Plates were incubated at 30 and 55°C for 1-2 days.

For the enumeration of bacterial spores, the homogenized sample suspension was heated at 80°C for 5 min. Serial dilutions were prepared from this heated suspension and mixed with PCA to which an additional top layer of 1.5% bacteriological agar (Oxoid, LP0011) was added to prevent spreading of colonies. Plates were incubated at both 30 and 55 °C for 2 days.

Lactic acid bacteria (LAB) were enumerated on MRSA (Oxoid CM0361) with 0.1% (w/v) natamycin (Delvocid, DSM, Delft, The Netherlands) to prevent yeast growth. Plates were incubated at 30°C for 72 h. To confirm the presence of presumed LAB, gram staining and catalase reactions were carried out. The results were corrected, based on counting of gram-positive and catalase negative organisms.

Enterobacteriaceae were counted with Violet Red Bile Glucose Agar (VRBGA; Oxoid CM0485). The medium was boiled twice before use. Plates were incubated at 37°C for 24 h. To confirm the presence of Enterobacteriaceae, oxidase- and glucose fermentation tests on presumptive colonies were carried out. The results were corrected based on the counting of oxidase negative, but glucose fermenting colonies.

Yeasts and moulds were enumerated on three different media, namely Malt Extract Agar (MEA; Oxoid CM0059), Dichloran-Glycerol Agar (DG18; Oxoid CM0097) and Rose Bengal Chloramphenicol Agar (RBCA; Oxoid CM0549), to which 100 mg/l chloramphenicol (Oxoid, SR0078E) was added. The diluted suspension (0.1 ml) was added to make spread plates which were incubated at 25°C for 2-4 days.

All enumerations of bacteria were carried out under both aerobic and anaerobic incubation conditions. Anaerobic growth conditions were achieved by flushing gas jars with a mixture of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen. All counts were made in duplicate for each of the duplicated samples and the results were reported as the means ± SD (n=4). From plates with 20-300 colonies, the square root of the total number of colonies was randomly selected as isolates.

DNA extraction and PCR amplification

Genomic DNA of bacteria was extracted by a Bacteria Genomic DNA Purification Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The 16S rRNA gene was amplified using forward primer 5'-AACGCGAAGAACCTTAC-3' and reverse primer 5'-CGGTGTGTACAAGACCC-3' (Niemann et al., 1997). PCR was performed with a total volume of 50 µl containing 26.6 µl ddH_2O, 5 µl PCR buffer, 3 µl $MgCl_2$ (25 mM), 10 µl dNTP (2 mM), 2 µl of each primer (10 µM), 1 µl DNA template, and 0.4 µl Taq DNA polymerase (5 U/µl) (Fermentas, USA). PCR was done using the GeneAmp PCR system

9700 (Applied Biosystems, USA) with the following PCR conditions: initial denaturation for 5 min at 94°C; 35 cycles each consisting of 30 s at 94°C, 20 s at 56°C, and 1 min at 72°C; and extension for 7 min at 72°C followed by cooling at 4°C. The PCR products were analysed by electrophoresis and stored at -20°C for further sequencing analysis.

Genomic DNA of fungi was extracted by a Yeast Genomic DNA Purification Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The 26S rRNA gene and ITS regions were amplified by different primer sets. For yeast strains, forward primer NL1 (5′-TGCTGGAGCCATGGATC-3′) and reverse primer RLR3R (5'-GGTCCGTGTTTCAAGAC-3') were used for amplification of the D1/D2 domain of the 26S rDNA gene; forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for amplification of the ITS 1 and ITS2 regions (Okoli et al., 2007). For moulds, forward primer V9G (5'-TTACGTCCCTGCCCTTTGTA-3') and reverse primer LS266 (5'-GCATTCCCAAACAACTCGACTC-3') were used for amplification of ITS1-5.8S-ITS2 (Zhang et al., 2011c). The PCR master mix was the same as described for bacteria, and PCR conditions were as follows: initial denaturation for 5 min at 95°C followed by 35 cycles comprising denaturation at 95°C for 60 s, annealing was at 52°C for 45 s, extension at 72°C for 60 s, and final 7 min extension at 72°C followed by 4°C. The PCR products were analysed as described before.

Sequencing and strain identification

The PCR products were purified using GFX columns (Amersham Pharmacia Biotech Inc., Roosendaal, The Netherlands). The bacterial and fungal rRNA genes were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems) and analysed on an ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA). The sequences were compared with those present in the GenBank/NCBI database using the BLAST 2.0 program (Altschul et al., 1990). Sequences were assembled and edited with Seqman II software (DNAStar Inc., Madison, Wis.) and aligned with Megalign (DNAStar Inc., Madison, Wis).

DNA extraction and PCR-DGGE analysis

DNA from *Fen-Daqu* samples was extracted according to Wang et al. (2008b) and

diluted to 1-50 ng/μL. Two sets of universal primers were tested for the amplification of fragments of the 16S rRNA and 26S rRNA genes, respectively. The V6-V8 region of the 16S rRNA gene was amplified using the primers EUB968GC-for and EUBL1401-rev (500 bp) (Nubel et al., 1996). The D1 region of the 26S rRNA gene was amplified using the primers NL1GC-for and LS2-rev (250 bp) (Flórez et al., 2006). All PCR reactions were carried out on an AG 223B1 Thermoblock (Eppendorf, USA). Amplifications were performed as described previously. The sizes and quantities of the PCR products were determined using 1.5% agarose gel electrophoresis. The amplification products obtained as described above were subjected to DGGE analysis using the Dcode Universal Mutation Detection system (Bio-Rad Laboratories, Richmond, CA, USA) on 20 cm × 16 cm × 1 mm gels. Electrophoresis was performed at 60°C in 0.5 × TAE buffer (20 mM Tris-acetate, 2 mM EDTA; pH 8.0) using 8% polyacrylamide gels containing 30-60% urea-formamide linear denaturing gradient (100% corresponded to 7 M urea and 40% v/v formamide) increasing in the direction of electrophoresis for 16 h at 85V. Following electrophoresis, the gels were stained by $AgNO_3$ solution as follows. The gel was fixed and shaken gently in 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 2 g/l $AgNO_3$ staining solution and shaken gently for 10 min, followed by a brief rinse 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. The gel was scanned with "Quantity One" software using a calibrated imaging densitometer GS-710 (BioRad, CA, USA). The identity of the microorganisms was revealed by sequencing selected bands from the DGGE profiles. Small fragments of selected DGGE bands were punched out from the gels, put in sterile water and boiled for 30 min to release DNA. The gel solutions were cooled and stored overnight. The obtained supernatant was used directly for re-amplification of the PCR products with primers described above without GC clamp.

Statistics

Statistical analysis was carried out using IBM-SPSS V19.0 (IBM® SPSS® Statistics; NY, U.S.A). A one-way ANOVA with Duncan's test was used to determine the significance of different microbial enumeration data.

4.3 Results

Enumeration of representative bacteria and fungi on selective media

The population levels of bacteria and fungi that were present in the inner and outer portions of the *Fen-Daqu* starter are summarized in Table 4.1. The total viable numbers of mesophilic and thermophilic bacteria in the inner and outer parts were not significantly different; whereas the numbers of bacterial spores were significantly higher in the inner part. The numbers of mesophilic bacteria, including total viable cells, LAB and Enterobacteriaceae, were similar when grown under aerobic or anaerobic conditions. However, total numbers of thermophilic bacterial spores were about 1 log CFU/g higher when grown aerobically, especially for the inner portion. The levels of LAB and Enterobacteriaceae were similar in both inner and outer portions of *Daqu*. The total counts of yeasts and moulds in inner *Daqu* were about 1 log CFU/g lower than in outer *Daqu*. No statistically significant differences were found for fungal counts when grown at 25°C or 37°C. We noticed that isolation using RBCA resulted in higher number of countable colonies if compared to the other two media used, viz. MEA and DG18. Bacteria were generally numerically dominant microorganisms in *Fen-Daqu*, followed by yeasts and moulds. The numbers of fungal colony forming units were more than 2 log CFU/g higher than those of LAB and Enterobacteriaceae.

Bacterial diversity based on identification of 16S rDNA

A total of 109 bacterial strains were randomly selected and identified by sequencing the 16S rDNA. Thirteen species were encountered in *Fen-Daqu*. The results are presented in Table 4.2. *Bacillus* spp. were predominant in *Fen-Daqu*. Particularly, *Bacillus licheniformis* and *Bacillus subtilis* together represented about 57% of the bacteria isolated from inner and outer portions of *Fen-Daqu*. In addition, *Brevibacterium* sp., LAB, i.e. *Enterococcus faecalis*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*, represented 4, 17, 6, and 6% of the total number of isolates, respectively. Other bacteria such as *Salmonella enterica* and *Escherichia coli* were also encountered in outer *Daqu*.

Table 4.1 Microbiota of inner and outer parts of *Fen-Daqu* presented as log CFU/g

log CFU/g	Aerobic counts		Anaerobic counts	
Microbial groups	inner	outer	inner	outer
Mesophilic bacteria	7.5 ± 0.4^{def}	7.5 ± 0.6^{def}	7.4 ± 0.2^{cdef}	7.7 ± 0.2^{ef}
Mesophilic bacterial spores	7.1 ± 0.6^{bcdef}	6.6 ± 0.2^{bcd}	7.7 ± 0.3^{ef}	6.8 ± 0.4^{bcdef}
Thermophilic bacteria	7.4 ± 0.1^{cdef}	6.9 ± 0.4^{bcdef}	7.5 ± 0.2^{cdef}	6.7 ± 0.5^{bcde}
Thermophilic bacterial spores	7.8 ± 0.3^{f}	6.7 ± 0.3^{bcde}	7.2 ± 0.2^{bcdef}	6.4 ± 0.5^{bc}
Lactic acid bacteria	4.0 ± 0.4^{a}	4.9 ± 0.3^{a}	4.0 ± 0.3^{a}	4.8 ± 0.2^{a}
Enterobacteriaceae	4.0 ± 0.6^{a}	4.8 ± 0.8^{a}	4.0 ± 1.2^{a}	4.8 ± 0.7^{a}
Fungi on MEA 25ºC	6.2 ± 0.7^{b}	7.1 ± 0.6^{bcdef}		
Fungi on MEA 37ºC	6.7 ± 1.2^{bcde}	6.9 ± 1.6^{bcdef}		
Fungi on DG18 25ºC	6.3 ± 0.5^{bcd}	7.1 ± 0.3^{bcdef}		
Fungi on DG18 37ºC	6.6 ± 0.7^{bcd}	7.1 ± 1.2^{bcdef}		
Fungi on RBCA 25ºC	6.7 ± 0.5^{bcde}	7.7 ± 0.6^{ef}		
Fungi on RBCA 37ºC	6.7 ± 0.5^{bcde}	7.6 ± 0.4^{def}		

Value represent means ± SD (n=4). Means with different superscripts are significantly different (One-Way ANOVA ; $P<0.05$).

Table 4.2 Bacterial diversity in *Fen-Daqu*

Species identification	Related GenBank sequence	% of bacterial isolates (n=109)	Isolated from[a]	Identity
Bacillus cereus	EU111736	1	O	380/380 (100%)
Bacillus licheniformis	AF399743	39	I & O	393/393 (100%)
Bacillus pumilus	EU874880	1	I	387/392 (98.7%)
Bacillus subtilis	FJ225312	18	I & O	392/392 (100%)
Brevibacterium sp.	EU596384	4	O	396/396 (100%)
Escherichia coli	EU026432	1	O	364/365 (99.7%)
Enterococcus faecalis	AB507170	17	I & O	389/391 (99.5%)
Lactobacillus plantarum	FJ749885	6	O	362/363 (99.7%)
Leuconostoc citreum	FJ040203	1	O	364/366 (99.5%)
Micrococcus luteus	FJ380953	1	O	381/382 (99.7%)
Pediococcus pentosaceus	FM179609	6	I & O	352/352 (100%)
Pseudomonas aeruginosa	GQ180118	2	O	369/370 (99.7%)
Salmonella enterica	FJ997268	3	O	343/343 (100%)

n = the total number of isolates
[a] I/O: isolated from Inner or Outer part of *Fen-Daqu*.

Fungal diversity based on identification of D1/D2 domain of 26S rDNA and ITS region

A total of 81 fungal strains were randomly selected and identified by rDNA sequencing as shown in Table 4.3. *Absidia corymbifera*, *Aspergillus flavus*, *Rhizopus stolonifer* and *Saccharomycopsis fibuligera* occurred throughout the inner and outer portions of *Fen-Daqu*. Others were found only in the outer portion, namely *Mucor circinelloides*, *Penicillium commune*, *Rhizomucor variabilis* var. *regularior*, *Pichia kudriavzevii* (formally known as *Issatchenkia orientalis*), *Wickerhamomyces anomalus* (formally known as *Pichia anomalus*) and *Saccharomyces cerevisiae*. One species, *Rhizomucor pusillus* (1%), was isolated from the inner portion only. The predominant isolates consisted of *Sm. fibuligera* (56%), *Abs. corymbifera* (11%), *W. anomalus* (8%), *P. kudriavzevii* (6%) and *R. stolonifer* (6%).

Analysis of bacterial and fungal populations using Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE analysis of the amplified 16S rDNA fragments obtained from the samples of the inner and outer portions of *Fen-Daqu* provided the fingerprint shown in Figure 4.2. Up to twelve bands, representing 10 different species were detected in the polyacrylamide gel. *Bacillus* spp., *Lactobacillus* spp. and *Weissella* spp. were the dominant bacterial species. A higher bacterial diversity was found in the outer layers of *Daqu* when compared to the inner part, as evidenced by the presence of *Staphylococcus saprophyticus*, *Lactobacillus sanfranciscensis* and *Bacillus megaterium* in the outer layers. DGGE fingerprints of the amplified fungal 26S rDNA fragments with primers NL1GC and LS2 are shown in Figure 4.3. The identified microbiota in the *Fen-Daqu* was less complex than the bacterial biota. Only four fungal species were detected, namely *W. anomalus*, *Sm. fibuligera*, *P. kudriavzevii* and *Debaromyces hansenii.* The fungal biota of inner and outer *Daqu* layers were largely similar.

Table 4.3 Fungal diversity in *Fen-Daqu*

Group of Fungi	Species identification	Related GenBank sequence	% fungal population (n=81)	Isolated from[a]	Identity
Moulds	*Absidia corymbifera*	AB305110	11	I/O	757/758 (99.9%)
	Aspergillus flavus	FJ878681	4	I/O	651/655 (99.4%)
	Mucor circinelloides	DQ118990	1	O	638/638 (100%)
	Penicillium commune	AF236103	3	O	669/676 (99.0%)
	Rhizomucor pusillus	AB369914	1	I	605/606 (99.8%)
	Rhizomucor variabilis var. regularior	DQ119007	2	O	528/530 (99.6%)
	Rhizopus stolonifer	DQ273817	6	I/O	242/244 (99.2%)
Yeasts	*Pichia kudriavzevii*	AY939808	6	O	547/547 (100%)
	Saccharomyces cerevisiae	EU798694	2	O	652/654 (99.7%)
	Saccharomycopsis fibuligera	FJ475057	56	I/O	728/728 (100%)
	Wickerhamomyces anomalus	EF449518	8	O	558/558 (100%)

n = the total number of isolates
[a] I/O: isolated from Inner or Outer part of *Fen-Daqu*.

4.4 Discussion

The bacteria of *Fen-Daqu* represent higher numbers than the yeasts and moulds. It should be realised however that since yeast cells are larger than bacterial ones, this doesn't imply that bacteria have a larger metabolic impact on the eco-physiology or biochemistry of *Daqu*. Studies on other types of *Daqu*, i.e. light-flavour and strong-flavour *Daqu* types (Leimena, 2008; Qiao et al., 2004; Zhang, 1999) also showed the presence of high numbers of bacteria. This high level (7-8 log CFU/g) of especially thermophilic bacteria and spores in *Daqu* makes this starter different from other Asian traditional alcoholic fermentation starters such as *men* (Dung et al., 2007; Thanh et al., 2008) or *ragi* (Hesseltine et al., 1988) of which the bacterial loads of 2.6-6.2 log CFU/g and 4.3-5.8 log CFU/g, respectively, represented mainly lactic acid bacteria.

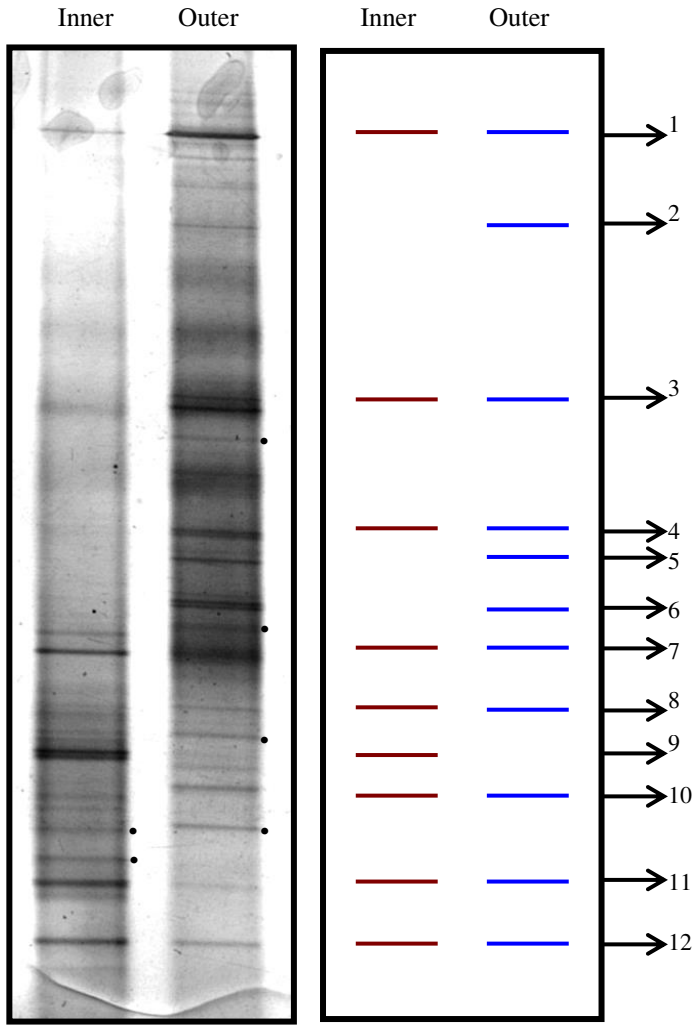

Figure 4.2 DGGE profiles (30-60% denaturant) representing bacterial 16S rRNA gene fragments of *Fen-Daqu* samples. 1, *Weissella cibaria*; 2, *Staphylococcus saprophyticus*; 3, *Enterobacter aerogenes*; 4, *Lactobacillus sanfranciscensis*; 5, *Lactobacillus lactis*; 6, *Bacillus megaterium*; 7, *Lactobacillus plantarum*; 8, *Weissella confusa*; 9, *Uncultured bacterium*; 10, *Bacillus licheniformis*; 11, *Weissella cibaria*; 12, *Weissella confusa*. The dotted bands had also been excised but could not be identified since no amplicons were obtained.

Combining the results of culture-dependent and -independent analyses, we found that the outer layers of *Fen-Daqu* have a broader microbial biodiversity and higher numbers of mesophilic microorganisms than the inner part, which contained less species and higher

numbers of thermophilic microorganisms, mostly *Bacillus* spp. This observation is consistent with those of previous studies (Shi et al., 2009; Wang et al., 2008).

This "microbiota stratification" within the blocks of *Daqu* may be explained by the changes of temperature that take place during the processing of *Fen-Daqu* (Zheng et al., 2011), as well as the characteristics of the solid state fermentation of *Daqu*, which results in mass - and heat gradients causing relatively high temperatures in the centre (inner portion) of the blocks (data not shown).

Fen-Daqu is a representative of light-flavour *Daqu*. During its manufacture by solid-state fermentation, the maximum temperature in the centre of the *Daqu* blocks during fermentation does not exceed 50°C (Kang, 1991). Most bacteria tolerate these temperatures and, therefore, a wide range of bacterial species was observed in this type of *Daqu*, particularly at the somewhat cooler outside. *B. subtilis* and *B. licheniformis* were the dominant members from the inner portion. Bacterial DGGE patterns also revealed the presence of *Weissella cibaria* and *Weissella confusa*. Interestingly, the total viable counts in the inner part of *Daqu* were sometimes lower than those of bacterial spores present, which may have been caused by heat-activation of spores during the preparatory heat treatment at 80˚C for 5 min before plating (Morn et al., 1990). We note that spore-forming bacteria are the predominant microbiota of *Fen-Daqu*, especially in the inner part.

The analyses of microbial communities in *Fen-Daqu* were carried out by both culture-dependent and -independent approaches. The *Bacillus* spp. encountered in *Fen-Daqu* were *B. licheniformis, B. subtilis, B. pumilus*, *B. megaterium* and *B. cereus.* Of these, *B. licheniformis* was most prevalent with 39% strains identified and the species was detected by both approaches. *B. subtilis* was another dominant species, with 18% identified strains, but it could not be detected by DGGE. In comparison, the method used by Shi et al (2009) encountered two *Bacillus* spp. in *Fen-Daqu* namely *B. licheniformis* and *Bacillus decisifrondis*.

The functionality of the bacterial biota needs further investigation. *B. subtilis*, *B. licheniformis* and *B. pumilus* may facilitate the conversion of starch into fermentable carbohydrates due to their amylolytic activity, thus generating a suitable substrate for the second stage of liquor production, i.e., the alcoholic fermentation (Mukherjee et al., 2009;

Wang et al., 2008a). In addition, *Bacillus* spp. produce nitrogenous flavour compounds such as diverse pyrazines (Li et al., 2011; Zhang et al., 2011c; Zheng et al., 2011) and we expect that these to contribute to *Daqu* flavour.

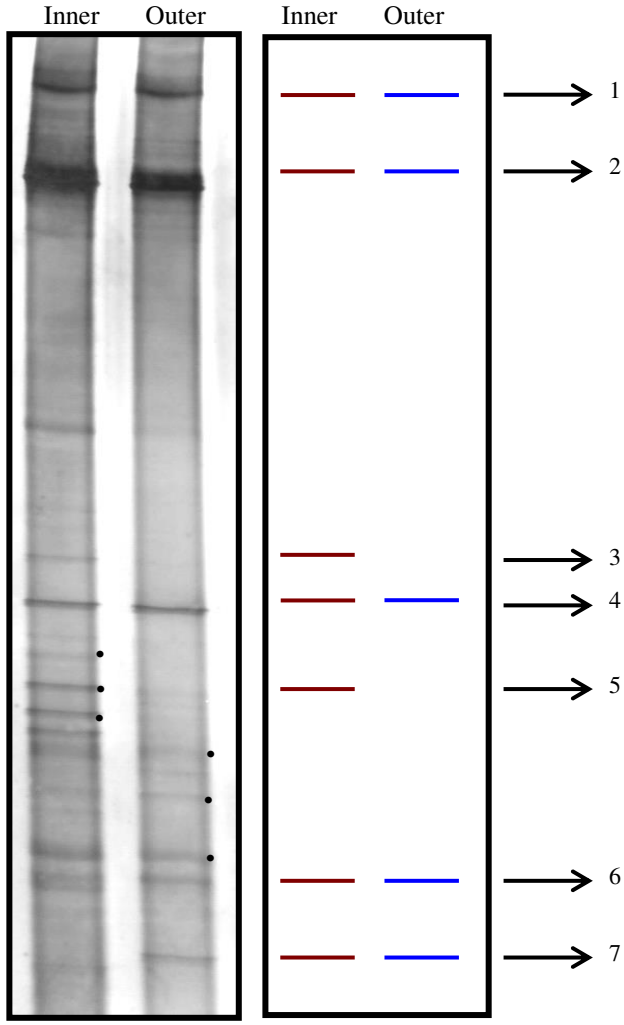

Figure 4.3 DGGE profiles (30-65% denaturant) representing 26S rRNA gene fragments of *Fen-Daqu* samples. 1, *Pichia kudriavzevii*; 2, *Saccharomycopsis fibuligera*; 3, *Wickerhamomyces anomalus*; 4, *Debaryomyces hansenii*; 5, *Pichia kudriavzevii*; 6, *Wickerhamomyces anomalus*; 7, *Pichia kudriavzevii*. The dotted bands had also been excised but could not be identified since no amplicons were obtained.

Several lactic acid bacteria viz., *Ent. faecalis, Ped. pentosaceus, Lb. plantarum, Lb, lactis, Leuc. citreum*, *Ws. cibaria* and *Ws. confusa* were identified that commonly occur in fermenting matter (Nout, 2009). Their contribution to the flavour development of *Fen-Daqu* or the final fermented liquor, for example, due to the formation of organic acids and other flavour compounds (Huang et al., 1993; Li et al., 2005; Wee et al., 2008) requires further attention.

Other bacteria including *E. coli*, *Ps. aeruginosa*, *M. luteus*, and *Sa. enterica* occurred in low numbers. Although we did not study their effects on quality or safety of *Daqu* we suggest that, because of their low numbers, they are not essential for the fermentation process, and that they originate from external contamination sources, such as air, soil, hands, or insects. Their presence, however, is not expected to pose a safety problem, as after fermentation, the product will be distilled to obtain the final liquor. During the steam distillation the cells of bacteria and fungi will be killed. Moreover, the alcohol concentration of the crude distillate is approximately 70% v/v, which is adequate to practically sterilize the liquor.

In this study we used three mycological media (MEA, DG18 and RBCA) (Boekhout et al., 2003) to increase the detection of diverse fungal biota. In *Daqu* analysis, RBCA was superior for yeasts and moulds enumeration, mainly because distinct individual colonies were obtained and spreading of mould colonies was inhibited due to the presence of rose bengal (Baggerman, 1981). DG18 proved most appropriate to distinguish between yeasts and moulds, although yeasts were occasionally overgrown by spreading fungi, such as *Mucor* spp.

In contrast with the distinct bacterial population, the yeast microbiota identified from *Fen-Daqu* is more similar to that described from other Asian traditional alcoholic starters (Hesseltine et al., 1988; Jeyaram et al., 2008; Saelim et al., 2008; Sujaya et al., 2004; Tsuyoshi et al., 2005). These studies revealed that *Sm. fibuligera* occurred commonly in these starters, and this species plays an important role during the initial stages of alcoholic fermentation. *Sm. fibuligera* typically grows prior to the main alcoholic fermentation and produces various enzymes, particularly glucoamylase and α-amylase, which contribute to glucose accumulation (Brimer et al., 1998; Horváthová et al., 2004; Knox et al., 2004;

Lemmel et al., 1980; Steverson et al., 1984). It was also found that glucoamylase produced by *Sm. fibuligera* can digest native starch (Chi et al., 2009), which improves the degradation of starch from the raw materials (i.e. barley and pea) of *Daqu*. *S. cerevisiae* usually dominates in alcoholic fermentations (Li et al., 2011; Nout, 2009; Urso et al., 2008) as it has the ability to grow under strictly anaerobic conditions. However, in the DGGE profiles, *S. cerevisiae* was not detected and using isolation only one strain of *S. cerevisiae* was obtained. This demonstrates that *S. cerevisiae* is not a dominant yeast species in *Daqu*. Recently, a study of Li et al (2011) investigated the species that involved in-situ *Fen*-liquor fermentation. This result indicated that the major active yeast species during this fermentation process was *S. cerevisiae*. The dominance of *S. cerevisiae* in alcoholic fermentation might be due to its competitive growth in the presence of fermentable sugars and its ethanol tolerance. We expect that it could grow quickly and become dominant during the liquor fermentation stage, such as has been observed in various wine fermentations (Dung et al., 2006; Dung et al., 2007; Jeyaram et al., 2008; Nyanga et al., 2007; Sujaya et al., 2004). Or it might be the host yeast species recycled in the fermentation jars. Mohanty et al. (2009) investigated the effect of different environmental factors on the *S. cerevisiae* fermentation. They observed that the combination of higher temperatures and lower moisture contents decreasing to around 10% at the end of maturation. Such conditions could be expected to limit the growth and survival of *S. cerevisiae.* This may thus well explain the low numbers of *S. cerevisiae* in matured *Daqu*. Other yeasts, such as *P. kudriavzevii* and *W. anomalus*, were observed in *Daqu* as well by both detection approaches. These yeast species are common in cereal fermentations (e.g. *men*, *hamei*, wheat *qu* and *zaopei*) and in combination with LAB, have been associated with the production of flavour and ethanol (Dung et al., 2006; Jeyaram et al., 2008; Nout, 2009; Thanh et al., 2008; Xie et al., 2007; Zhang et al., 2007a).

Six genera of moulds, viz., *Absidia*, *Aspergillus*, *Mucor, Penicillium*, *Rhizomucor* and *Rhizopus* were obtained by direct plating. The predominant moulds in *Fen-Daqu* were *A. corymbifera* and *R. stolonifer* (Mucoraceae), which are known to be strong amylase producers (Blandino et al., 2003; Hesseltine et al., 1988; Thanh et al., 2008). *M. circinelloides*, *Rhizomucor variabilis var. regularior*, *R. pusillus* and *P. commune* were also

isolated. In other fermentations, these moulds were responsible for volatile production during fermentation, such as ethanol, 2-methyl-1-butanol and 3-methyl-1-butanol (Sunesson et al., 1996; Sunesson et al., 1995; Wang et al., 2008a). In our analysis of *Fen-Daqu*, *Asp. flavus* was found as well. This species is known to produce aflatoxins (Degola et al., 2007) and, therefore, it may be prudent to check for the presence of mycotoxins in *Daqu* and derived products. Sometimes, the DGGE method using total DNA extracted from complex food products is unable to detect some fungal species, whereas these could be detected by culturing and identification by DNA sequencing. Cocolin (2001) and Prakitchaiwattana et al. (2004) reported that individual species in a mixed population could be detected by DGGE when the concentrations were higher than 4 log CFU/g, which may be the threshold level to detect species. *Fen-Daqu* is a kind of solid cake covered with mycelia of white moulds that mainly belong to the so-called zygomycetes. It seems that DNA of this group of fungi is more difficult to extract, even when pure cultures are used (Hrncirova et al., 2010). We assume that the absence of bands for this group fungi in our DGGE gel is mainly caused either by (i) inadequate DNA extraction of filamentous fungi from the complex food matrix of *Daqu*, or (ii) by the presence of high concentrations of competing DNA, such as that from yeasts in *Fen-Daqu.*

Presently, the study of microbial diversity can be undertaken with culture-dependent and/or culture-independent analyses. Both approaches have their advantages and disadvantages. From our study, we conclude that the culture-dependent analysis of *Daqu* samples resulted in a different and more complex microbiota than did DGGE analysis. A clear advantage of culturing is that a collection of pure cultures is obtained that can be used for further experimentation. A number of species (*B. licheniformis*, *Lb. plantarum, Sm. fibuligera, P. kudriavzevii* and *W. anomalus)* were detected using both approaches, that in total revealed 30 species, most of which were found by either the culture-dependent or culture-independent technique. This shows that although PCR-DGGE analysis provides a broad picture of the different groups of microorganisms present in *Daqu*, including uncultivable ones, a combined approach is preferred for the detection of dominant and minor species in order to better understand the complete microbial ecosystem present in such natural mixed fermentation starters.

Fen-Daqu is a representative of low-temperature *Daqu*. Although the manufacturing techniques have been modernized during recent years, the inconsistency and instability of *Daqu* is still a problem that hampers the standardization of Chinese liquor making. Our study provides qualitative and quantitative information on the microbial diversity present in *Fen-Daqu*. The knowledge of microbial composition and functionality will enable further upgrading of the Chinese traditional *Daqu* making processes, e.g. by selection of functionally important strains, and optimization of microbial composition and quality control.

Acknowledgements

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Chapter 5

Microbiota dynamics related to environmental conditions during the fermentative production of *Fen-Daqu*

Abstract

Chinese *Daqu* is used as a starter for liquor and vinegar fermentations. It is produced by solid state fermentation of cereal-pulse mixtures. A succession of fungi, lactic acid bacteria and *Bacillus* spp. was observed during the production of *Daqu*. Mesophilic bacteria followed by fungi, dominated the first phase of fermentation. Next, lactic acid bacteria increased in relative abundance, resulting in an increase of the acidity of *Daqu*. At the final stages of fermentation, *Bacillus* spp. and thermophilic fungi became the dominant groups, possibly due to their tolerance to low water activity and high temperature. Both culture-dependent and culture-independent analyses confirmed that *Bacillus* spp. were ubiquitous throughout the process. Yeast species such as *Wickerhamomyces anomalus*, *Saccharomycopsis fibuligera* and *Pichia kudriavzevii* were present throughout almost the entire fermentation process, but the zygomycetous fungus *Lichtheimia corymbifera* proliferated only during the final stages of fermentation. Canonical correspondence analysis (CCA) revealed the significance of acidity, moisture content and temperature in correlation with the composition of the microbial communities at different stages.

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. International Journal of Food Microbiology 182-183, 57-62.

5.1 Introduction

Chinese liquor (a traditional distilled alcoholic beverage) and vinegar are important commercial fermented products in China with an annual production of about 12 million metric ton and 5 million metric ton in the country, respectively (Han, 2007). *Daqu* serves as a fermentation inoculum, and it makes a considerable contribution to the flavour of Chinese liquor and traditional Chinese vinegar (Wu et al., 2009a). It is an intermediate natural fermentation product that contains metabolically active microorganisms and enzymes. It is an essential ingredient responsible for the release of fermentable sugars from sorghum starch. Generally, *Daqu* can be classified into four major types, i.e. light-flavour *Daqu*, strong-flavour *Daqu*, sauce-flavour *Daqu* and miscellaneous-flavour *Daqu* (Zheng et al., 2011). *Fen-Daqu* is a light-flavour *Daqu* that is prepared from barley and peas in five steps: (i) formulation of ingredients; (ii) grinding and mixing; (iii) shaping; (iv) incubation (about 1 month); and (v) maturation (about 6 months). The incubation stage, also called the fermentation stage, as described by Zheng et al. (2012) can be divided into seven stages according to the core temperature profile of *Daqu* during its production (Figure 5.1): Stage 1 - *Woqu* (layering and covering, 20°C), Stage 2 - *Shangmei* (molding, 38°C), Stage 3 - *Liangmei* (cooling and hardening, 24-36°C), Stage 4 - *Chaohuo* (succession of dominant groups of microorganisms, 43-47°C), Stage 5 - *Dahuo* (enhancing microbial metabolism, 43°C), Stage 6 - *Houhuo* (evaporative dehydration and equilibration, 34-38°C), and Stage 7 - *Yangqu* (pre-maturation, 30°C).

Fen-Daqu is produced using traditional fermentation technology and contains microorganisms that are naturally present in the ingredients (i.e., barley and peas) and its production environment (i.e., tools, soil, air, and machines) (Lei, 2011). Recently, the microbial diversity in various types of *Daqu* has been investigated (Lei, 2011; Wang et al., 2011b; Zheng et al., 2012). However, limited data have been reported on the microbial communities prevailing during *Daqu* production (Li et al., 2013). With their study, only a culture-independent cloning method was used, and the microbial dynamics in relation with the environmental conditions during *Daqu* fermentation processes has not been reported. Therefore, the objectives of this study were to analyse changes in temperature, acidity, moisture content and microbial communities during *Daqu* production and to understand the

predominance and succession of microbes during its fermentation process as a function of dynamics of environmental conditions.

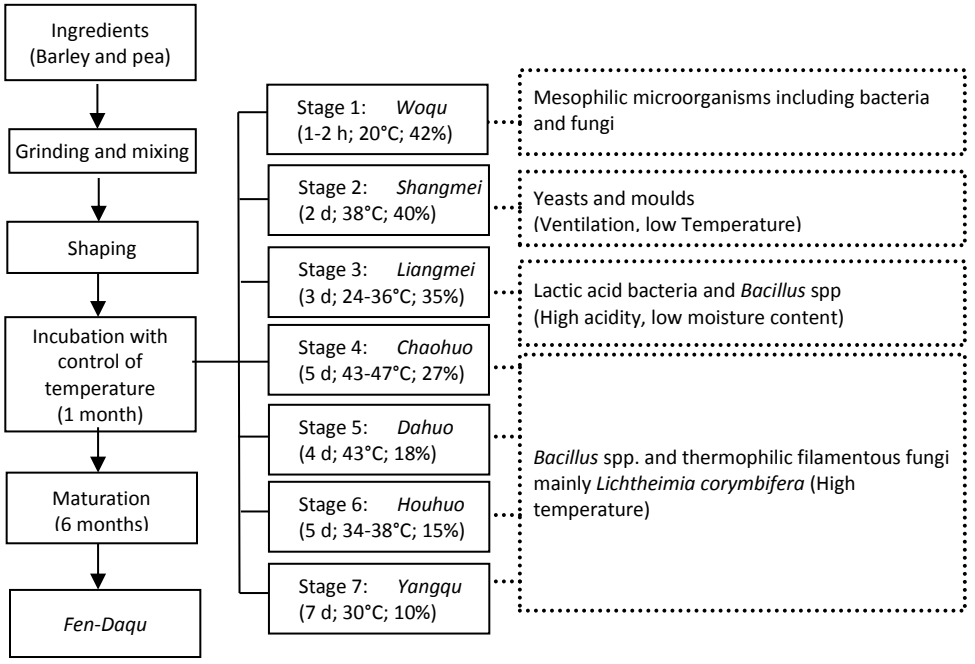

Figure 5.1 Process diagram for the production of *Fen-Daqu*.

Note: The text within the rectangle marked with black-dotted lines present the dominant microorganisms at each stage. The content given in brackets presents the factors that determine microbial diversity at that stage. The percentage (%) represents the moisture content in the stages.

5.2 Materials and Methods

Sampling

Fen-Daqu samples were obtained from Shanxi Xinghuacun Fenjiu Distillery Co. Ltd (Fenyang, Shanxi, China) during the month of October 2009, which is the season of *Daqu* production. Samples were separately taken from four independent processes and collected at the seven production stages, as shown in Figure 5.1. At each of the sampling events, approximately 100 g of *Daqu* was aseptically collected in triplicate (in order to obtain adequate representation, three blocks were randomly selected from upper, middle and lower layers), ground and pooled to provide an experimental *Daqu* powder sample. Samples were

then collected into sterile Stomacher bags (Stomacher® Lab System, London, UK), and transported to the laboratory in a cooler box.

Microbiological analysis - culture dependent methods

(1) Enumeration and isolation

Samples from all 7 stages, each weighing 10 g were transferred to Stomacher bags and homogenized with 90 ml sterile PPS (Peptone Physiological Salt) solution containing (g/l) peptone (Oxoid LP0034) 1, and NaCl 8.5, in a Stomacher Lab-blender 400. Portions (1 ml) of the diluted suspensions were plated on different selective agar media. Total aerobic bacteria and spores, lactic acid bacteria, Enterobacteriaceae and fungi were enumerated on Plate Count Agar (PCA; Oxoid CM035), MRSA (Oxoid CM0361), Violet Red Bile Glucose Agar (VRBGA; Oxoid CM0485) and Malt Extract Agar (MEA; Oxoid CM0059), respectively. These were incubated at different temperatures (30°C, 37°C and 55°C) for the isolation and enumeration (by recording the number of CFU) of specific groups of micro-organisms (Zheng et al., 2012).

(2) Extraction of DNA from pure culture

Single colony isolates for subsequent DNA extractions were obtained from the counted plates. The plates corresponding to a number of colonies less than or equal to 50, were selected to perform isolations. The square root of the total number of colonies was randomly picked up from both duplicate plates. After purification, isolates were grown under the same conditions as used previously for cultivation. Cells at stationary phase were collected by centrifugation at 9000 *g* for further extraction of total DNA. The genomic DNA of bacteria and fungi was extracted using a Bacteria Genomic DNA Kit (Tiangen, Beijing, China) and a Yeast Genomic DNA Kit (Tiangen, Beijing, China), respectively, according to the manufacturer's instructions. DNA concentration was measured by UV-Vis spectrometry (Unico, Shanghai) and diluted to a final concentration of approximately 10 ng/µl. The DNA solutions were stored at -20°C.

(3) Nucleotide sequence accession numbers

The primers used in this study are listed in Table 5.1. The 16S rRNA gene sequences of the bacteria in this study were deposited in GenBank under the accession numbers KJ526822-KJ526949; the 26S rRNA and ITS gene sequences of the yeasts were deposited

in GenBank under the accession numbers KJ526950-KJ526985 and KJ527033-KJ527069, respectively; the ITS gene sequences of the moulds were deposited in GenBank under the accession numbers KJ527009-KJ527032.

Microbiological analysis—culture-independent methods

(1) PCR-DGGE analysis and bands excision

Total DNA was extracted from *Daqu* powder by the same method used previously (Zheng et al., 2012). Two sets of universal primers were tested for the amplification of a fragment of the 16S rRNA gene and the 26S rRNA gene (Table 5.1). Amplifications, DGGE analysis and identification of bands of interest were performed as described by Zheng et al. (2012). The sequences of excised bands in this study were deposited in GenBank under the accession numbers KJ526994-KJ527008 for bacteria and the accession numbers KJ526986-KJ526993 for fungi.

Physicochemical analysis

(1) pH measurements

The pH was measured with a pin electrode of a PB-10 pH meter (Sartorius, Germany) inserted directly into the sample suspension (1 g/10 ml). From each stage, four samples were measured in duplicate. Means and standard deviations were calculated.

(2) Determination of acidity

The acidity of the samples was determined in suspensions containing 25 g of *Daqu* in 150 ml of CO_2-free distilled water that was titrated with a standard 0.1 M NaOH solution. Total titratable acidity was expressed as g lactic acid per kg dry matter. Means and standard deviations were calculated on all data.

(3) Determination of temperature and relative humidity

The surface temperature of *Daqu* was recorded with a calibrated mini infrared thermometer gun (UNI-T UT301A, Beijing, China) at the time of sampling. The relative humidity (RH) of the incubation room was recorded with a humidity/temperature logger (Testo 175-H2, Shanghai, China). The continuous online detection of the core temperature of *Daqu* and room temperature was performed with electronic temperature sensors (iButton, Maxim, USA) which were inserted into the centre of the *Daqu* blocks (Figure 5.2)

and stuck on the wall of incubation room. For this purpose, during stage 1, three *Daqu* blocks were randomly selected from each incubation room and labelled. The i-buttons recorded data at hourly intervals until the end of stage 7. Means and standard deviations were calculated.

Statistical analysis

The plate count numbers of the four experiments were log transformed, and the average and standard error of the mean were calculated. Statistical analysis was carried out using IBM-SPSS V19.0 (IBM, SPSS Statistics; NY, U.S.A). A one-way ANOVA with Duncan's test was used to determine the significance of different microbial enumeration data and part of physical data (pH and acidity). DGGE data and environmental data were analysed by Canonical Correspondence Analysis (CCA), using CANOCO 4.5 for Windows software (Biometris, the Netherlands). Before performing the CCA, DGGE profiles were first transformed numerically using Quantity One v4.62 software (Bio-Rad, USA) so that the relative abundance of each species could be expressed as the relative intensity of each band. The resulting intensities were then expressed as the fraction of each species of the total microbial abundance. The ordination triplot obtained by CCA approximated the weighted average of each species with respect to environmental variables, which were represented as arrows. The length of these arrows indicated the relative importance of that environmental factor in explaining variation in microbial profiles, while the angle between the arrows (environmental factor-environmental factor or environmental factor - species) indicated the degree to which they were correlated. The distance between samples indicates their similarity in microbial diversity, i.e. the closer, the more similar. Significance was tested by the distribution-free Monte Carlo test (199 random permutations).

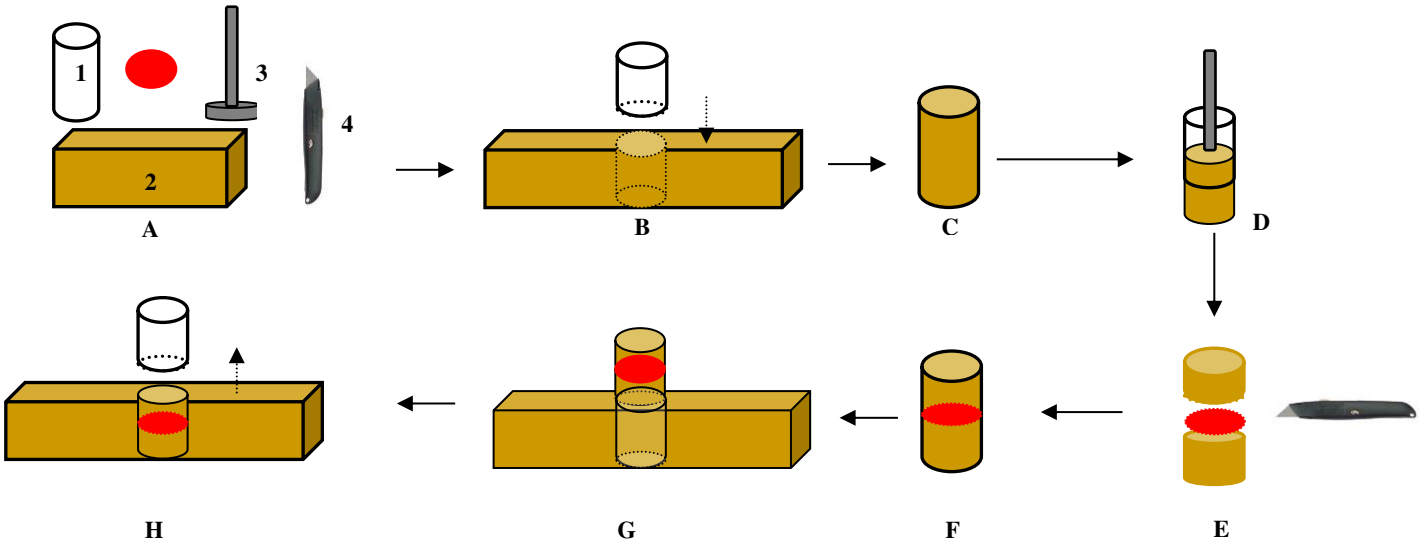

Figure 5.2 Insertion of temperature sensors into *Daqu*. Numbers refer to the different components (1: cylinder cutter with a 3 cm diameter; 2: *Daqu* block; 3: pusher; 4: knife; 5: electronic temperature sensor). Letters refer to the different steps (A: preparation of materials; B: insertion of cylinder cutter into *Daqu* block; C: taking cutter (*Daqu* included) out from *Daqu* block; D: pushing *Daqu* out; E: cut *Daqu* from the middle and insertion of temperature sensor into *Daqu*; F: putting cylinder cutter back to *Daqu*; G: returning *Daqu* to *Daqu* block; H: taking cutter out of *Daqu* block).

5.3 Results

Changes in viable cell counts over time during Daqu fermentation

Changes in microbial counts were monitored during the seven stages of *Fen-Daqu* fermentation (see Table 5.2). The average bacterial counts of the various groups at the start of fermentation (Stage 1) were rather low and varied between 3.4 log CFU/g and 6.2 log CFU/g with mesophilic bacteria dominant. After this stage, total counts increased over time, reaching values as high as 9-11 log CFU/g for mesophilic and thermophilic bacteria and bacterial spores, and 5-7 log CFU/g for Enterobacteriaceae and lactic acid bacteria. The levels of mesophilic and thermophilic bacteria and bacterial spores remained at the same level ($P< 0.05$) after Stage 5 (*Dahuo*). In the case of lactic acid bacteria, the highest numbers were obtained at stage 3 which decreased thereafter (Table 5.2). With respect to fungi, average levels of 5.2 to 8.1 log CFU/g were observed. The number of fungi increased approximately 3 orders of magnitude during Stage 2 (*Shangmei*) and showed a declining trend until the final stage 7 (*Yangqu*).

Identification of bacteria and fungi

A total of 112 bacterial isolates were obtained from the fermentation stages of *Fen-Daqu*. They were identified by conventional methods in combination with molecular methods. The bacteria were numerically dominant during the fermentation and could be classified as representatives of the genera *Bacillus*, *Lactobacillus*, *Acetobacter*, *Lactococcus* and *Staphylococcus* and of the family of Enterobacteriaceae (Table 5.3). Members of the genus *Bacillus* comprised approximately 70% of total bacterial isolates. By using a culture-based approach, *Bacillus* spp. were found during every stage of *Daqu* fermentation as predominant microorganisms with *Bacillus licheniformis* as the most common species. Eight different species of the genus *Bacillus* were isolated and identified during fermentation, i.e. *Bacillus anthracis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus curvatus/sakei*, *B. licheniformis*, *Bacillus megaterium*, *Bacillus pumilus* and *Bacillus subtilis.* Also the lactic acid bacterium *Pediococcus pentosaceus* was isolated frequently. Other species of lactic acid bacteria, such as *Lactococcus lactis*, *Lactobacillus plantarum*;

and *Staphylococcus epidermidis* were isolated only during one stage (stage 1, stage 3 and stage 7, respectively) and at relatively low numbers.

Table 5.1 PCR primers used in this study

Primer	Sequence (5′→3′)	Aims	Reference
B-for*	AGAGTTTGATCCTGGCTCAG	Amplification 16S rRNA gene and sequencing for bacteria	(Lima et al., 2012)
B-rev*	AAGGAGGTGATCCAGCCGCA		
NL1-for	GCATATCAATAAGCGGAGGAA AAG	D1 and D2 domains of 26S rRNA gene and sequencing for yeasts	(Zheng et al., 2012)
RLR3R-rev	GGTCCGTGTTTCAAGAC		
ITS5-for	GGAAGTAAAAGTCGTAACAAG G	Amplification of ITS1-5.8S-ITS2 gene and sequencing for yeasts	(Zheng et al., 2012)
ITS4-rev	TCCTCCGCTTATTGATATGC		
V9G-for	TTACGTCCCTGCCCTTTGTA	Amplification of ITS1-5.8S-ITS2 gene and sequencing for moulds	(Zheng et al., 2012)
LS266-rev	GCATTCCCAAACAACTCGACTC		
EUB968GC-for	CGCCCGGGGCGCGCCCCGGGCG GGGCGGGGGCAGGGGAACGCG AAGAACCTTAC	Bacterial DGGE	(Zheng et al., 2012)
EUBL1401-rev	CGGTGTGTACAAGACCC		
EUB968-for	AACGCGAAGAACCTTAC	Sequencing of excised bands from bacterial DGGE	(Zheng et al., 2012)
EUBL1401-rev	CGGTGTGTACAAGACCC		
NL1GC-for	CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGGCACGGGGCATAT CAATAAGCGGAGGAAAAG	Fungal DGGE	(Zheng et al., 2012)
LS2-rev	ATT CCC AAA CAA CTCGAC TC		
NL1-for	GCATATCAATAAGCGGAGGAA AAG	Sequencing of excised bands from fungal DGGE	(Zheng et al., 2012)
LS2-rev	ATT CCC AAA CAA CTCGAC TC		

The GC clamp is underlined.
* for: forward; rev: reverse

A total of 95 fungal isolates were obtained during the fermentation of *Fen-Daqu* and were identified by conventional microbiological analysis and sequencing of the gene encoding 26S rRNA (Table 5.3). Four yeast species were isolated; *Pichia kudriavzevii* and *Wickerhamomyces anomalus* were detected in six stages and seven stages, respectively. Among the filamentous fungi, the zygomycetous species *Absidia corymbifera* appeared during stage 5 and increased in relative abundance towards the end of the fermentation.

Bacterial DGGE patterns and identification of part of the obtained bands are shown in Figure 5.3. Up to 15 bands, representing 12 different species were detected during fermentation, of which six species (*Enterobacter aerogenes*, *Lactobacillus sanfranciscensis*, *Staphylococcus saprophyticus*, *Weissella cibaria*, *Weissella confusa* and uncultured bacterium) were not found by culture-dependent techniques. DGGE analysis confirmed the high relative abundance of *Bacillus* spp. throughout the fermentation process. Particularly, *B. licheniformis* (bands No. 9 and 15) was present throughout almost the entire process. At stage 1, 10 bands representing 8 different species were present, which indicates a high degree of diversity in the microbial community. From this stage onward, some bands disappeared, with mostly new bands emerging for both mesophilic and thermophilic bacterial species.

Apart from bacilli, also *Ws. cibaria* and *Ws. confusa* were dominant during *Daqu* fermentation and were present in different ratio during each stage of fermentation. Other lactic acid bacteria, such as *Lb. sanfranciscensis* and *Lb. plantarum*, were present in high ratio during the stage 2 and stage 1, respectively, and decreased during the final stages (stage 4 to stage 7).

By combining the results of culture-dependent and -independent methods, 23 bacterial species in total were observed in *Daqu*. Of these, 11 species (for example *Acetobacter pasterianus*, *Bacillus anthracis* and *Lc. lactis*) were detected only by culture-based methods. The highest bacterial diversity and the highest *Bacillus* spp. diversity were all observed during stage 3.

In general, four dominant bands occurred in the DGGE profile of the fungal communities (Figure 5.4). With *Saccharomycopsis fibuligera* and *Debaryomyces hansenii* as exceptions, all other species were also found using culture-based techniques. *Sm. fibuligera* occurred during all stages, except for stage 3 and *P. kudriavzevii* was encountered only after stage 1. *W. anomalus* was present throughout the entire *Daqu* fermentation process.

Table 5.2 Changes of viable microbial counts (log CFU/g), pH, and acidity during fermentation of *Fen-Daqu*

	Fermentation stages*						
	Stage 1	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Stage 6**	**Stage 7**
TMAB (30°C)	6.2 ± 0.1^{a}	8.2 ± 0.3^{b}	9.6 ± 0.1^{c}	8.5 ± 0.1^{b}	10.8 ± 0.4^{d}	10.4 ± 0.9^{d}	10.4 ± 0.3^{d}
TTAB (55°C)	3.5 ± 0.3^{a}	5.2 ± 1.3^{b}	5.3 ± 1.1^{b}	7.2 ± 1.0^{c}	8.1 ± 0.3^{cd}	8.6 ± 0.6^{d}	8.5 ± 0.5^{cd}
Mesophilic aerobic bacterial endospore (30°C)	4.1 ± 0.5^{a}	5.9 ± 0.4^{b}	5.1 ± 0.2^{b}	7.3 ± 0.2^{c}	9.6 ± 1.0^{e}	8.7 ± 0.6^{d}	8.8 ± 0.6^{de}
Thermophilic aerobic bacterial endospore (55°C)	3.7 ± 0.1^{a}	5.9 ± 0.4^{b}	5.9 ± 0.4^{b}	7.4 ± 1.1^{c}	8.4 ± 0.4^{cd}	8.8 ± 0.3^{d}	8.5 ± 0.4^{cd}
Lactic acid bacteria	4.9 ± 0.3^{a}	5.7 ± 0.2^{bc}	6.5 ± 0.1^{d}	6.3 ± 0.1^{cd}	6.1 ± 0.7^{cd}	6.0 ± 0.5^{cd}	5.3 ± 0.6^{ab}
Enterobacteriaceae	3.4 ± 0.2^{a}	3.8 ± 0.4^{ab}	4.2 ± 0.8^{bc}	5.0 ± 0.2^{d}	4.7 ± 0.2^{cd}	4.6 ± 0.5^{cd}	4.1 ± 0.5^{bc}
Fungi	5.2 ± 0.4^{a}	8.1 ± 0.4^{d}	7.7 ± 0.2^{cd}	7.3 ± 0.1^{bc}	7.4 ± 0.3^{bc}	7.4 ± 0.5^{bc}	7.2 ± 0.3^{b}
pH	4.3 ± 0.1^{a}	4.2 ± 0.1^{a}	4.4 ± 0.3^{a}	6.1 ± 0.6^{b}	6.8 ± 0.1^{c}	6.8 ± 0.1^{c}	6.9 ± 0.1^{c}
Acidity (g lactic acid/kg dry matter)	1.4 ± 0.2^{a}	4.6 ± 0.5^{d}	4.2 ± 0.3^{d}	3.7 ± 0.4^{c}	3.1 ± 0.7^{bc}	3.1 ± 0.4^{b}	3.3 ± 0.5^{bc}

TMAB: Total mesophilic aerobic bacteria
TTAB: Total thermophilic aerobic bacteria
Value represent means ± SD (n=4). Means with different superscripts are significantly different horizontally (One-Way ANOVA; $P < 0.05$).
* Stage 1: *Woqu*; Stage 2: *Shangmei*; Stage 3: *Liangmei*; Stage 4: *Chaohuo*; Stage 5: *Dahuo*; Stage 6: *Houhuo*; Stage 7: *Yangqu*

Table 5.3 Microbiota composition at each stage of *Fen-Daqu* fermentation

		Fen-Daqu fermentation stages*						
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7
Bacteria	*A. pasterianus*		[11.8]+ (I)				[6.25]+ (I)	
	A. tropicalis			[5]+ (I)				
	B. anthracis/B. cereus	+ (D)		[15]+ (I)		+ (D)	+ (D)	+ (D)
	B. amyloliquefaciens						[12.5]+ (I)	
	B. circulans			[5]+ (I)				
	B. licheniformis	[37.5]+ (I/D)	[23.5]+ (I/D)	[15]+ (I/D)	[81.25]+ (I/D)	[88.2]+ (I/D)	[43.75]+ (I/D)	[50]+ (I/D)
	B. megaterium	+ (D)		[15]+ (I/D)		+ (D)		+ (D)
	B. pumilus	[6.25]+ (I)	[11.8]+ (I/D)	[10]+ (I)			[25]+ (I)	
	B. subtilis	+ (D)	[23.5]+ (I)	+ (D)				[25]+ (I/D)
	Enterobacter sp./ *Escherichia* sp.	[25]+ (I)	[23.5]+ (I)	[15]+ (I)				
	E. aerogenes	+ (D)		+ (D)	+ (D)	+ (D)		
	Georgenia sp.	[6.25]+ (I)						
	Lb. curvatus/Lb. sakei		[5.8] + (I)					
	Lb. plantarum	+ (D)		[10]+ (I)	+ (D)	[11.8]+(/ID)	+ (D)	+ (D)
	Lb. sanfranciscensis		+ (D)		+ (D)	+ (D)	+ (D)	
	Lc. lactis	[12.5]+ (I)						
	Microbacterium sp.			[5]+ (I)				
	Ped. pentosaceus	[12.5]+ (I)		[5]+ (I)	[18.75]+ (I)		[12.5]+ (I)	[12.5]+ (I)
	St. epidermidis							[12.5]+ (I)
	St. saprophyticus	+ (D)			+ (D)	+ (D)	+ (D)	+ (D)
	Uncultured bacterium		+ (D)	+ (D)		+ (D)	+ (D)	+ (D)
	Ws. cibaria	+ (D)	+ (D)	+ (D)	+ (D)	+ (D)	+ (D)	+ (D)
	Ws. confusa	+ (D)	+ (D)	+ (D)	+ (D)	+ (D)	+ (D)	+ (D)
Yeasts	*D. hansenii*					+ (D)	+ (D)	
	P. kudriavzevii		[38.4]+ (I/D)	[23.1]+ (I/D)	[28.5]+ (I/D)	[8.3]+ (I/D)	[25]+ (I/D)	[28.5]+ (I/D)
	Pseudozyma sp.	[7.7]+ (I)						

Table 5.3 Microbiota composition at each stage of *Fen-Daqu* fermentation (continued)

		Fen-Daqu fermentation stages*						
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7
Yeast	*Sm. fibuligera*	+ (D)	+ (D)		+ (D)	+ (D)	+ (D)	+ (D)
	W. anomalus	[46.1]+ (I/D)	[23]+ (I/D)	[69.2]+ (I/D)	[71.4]+ (I/D)	[66.7]+ (I/D)	[37.5]+ (I/D)	[28.5]+ (I/D)
Filamentous fungi	*Alternaria* sp.			[7.6]+ (I)				[7.2]+ (I)
	Asp. versicolor						[6.2]+ (I)	
	Cladosporium sp	[23.1]+ (I)						
	L. corymbifera					[25]+ (I)	[25]+ (I)	[35.8]+ (I)
	Penicillium sp.	[15.3]+ (I)						
	Phoma sp	[7.7]+ (I)						
	Rhizomucor pusillus						[6.2]+ (I)	
	R. stolonifer		[38.4]+ (I)					

* Stage 1: *Woqu*; Stage 2: *Shangmei*; Stage 3: *Liangmei*; Stage 4: *Chaohuo*; Stage 5: *Dahuo*; Stage 6: *Houhuo*; Stage 7: *Yangqu*

+ indicates species is detected at this stage; I indicates strain obtained only by culturing at this stage; D indicates strain obtained only by DGGE at this stage; I/D indicates strain obtained from both isolation and DGGE at this stage. *A*: *Acetobacter*; *B*: *Bacillus*; *E*: *Enterobacter*; *Lb*: *Lactobacillus*; *Lc*: *Lactococcus*; *Ped*: *Pediococcus*; *St*: *Staphylococcus*; *Ws*: *Weissella*; *D*: *Debaryomyces*; *P*: *Pichia*; *Sm*: *Saccharomycopsis*; *W*: *Wickerhamomyces*; *L*: *Lichtheimia*; *Asp*: *Aspergillus*; *R*: *Rhizopus*; [ ] indicates the frequency of presence (%) of each species within the groups of bacteria or within the groups of yeasts and moulds at each stage of fermentation.

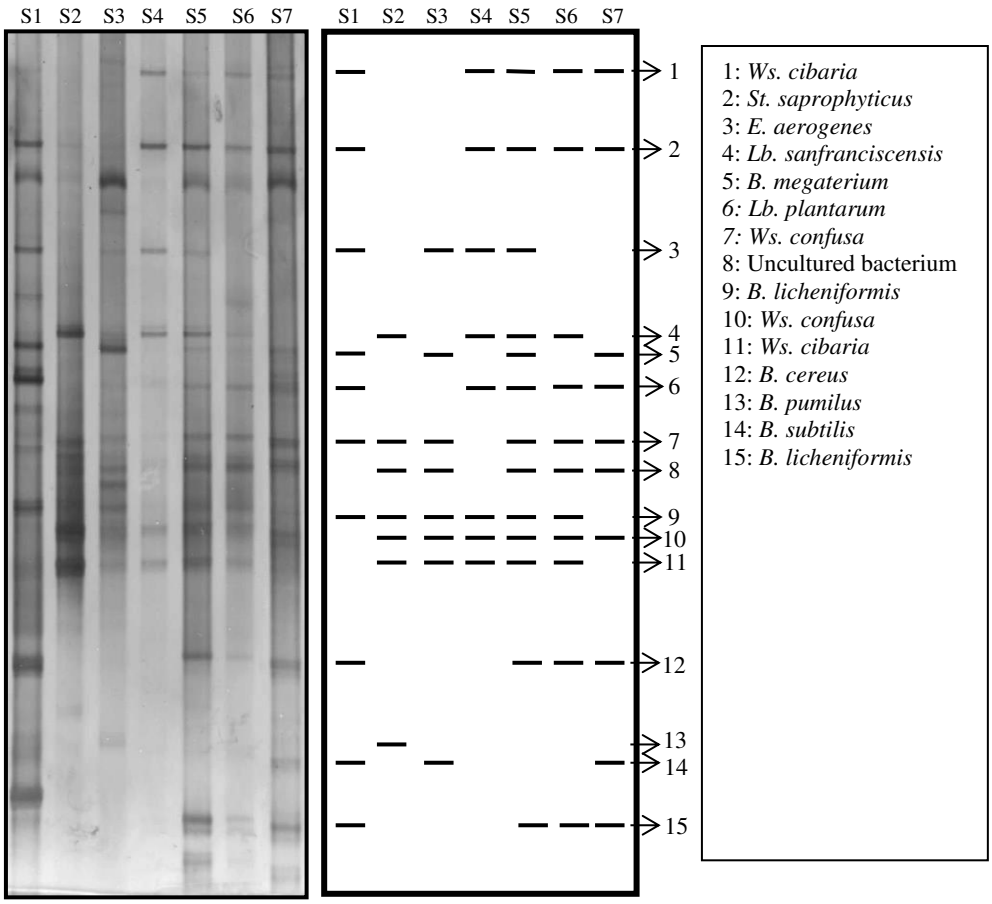

Figure 5.3 DGGE profiles (30-60% denaturant) representing 16S rRNA gene fragments of *Fen-Daqu* samples during fermentation (S1: Stage 1-*Woqu*; S2: Stage 2-*Shangmei*; S3: Stage 3-*Liangmei*; S4: Stage 4-*Chaohuo*; S5: Stage 5-*Dahuo*; S6: Stage 6-*Houhuo*; S7: Stage 7-*Yangqu*).

Changes in environmental factors during fermentation

The pH of *Daqu* increased steadily after stage 3 of the fermentation process, the titratable acidity increased rapidly during stage 2, reaching the maximum value of approximately 5 g/kg at the end of this stage, followed by a decline (Table 5.2).

Changes in core and surface temperatures of *Daqu* blocks and of the incubation room, as well as relative humidity and moisture content that occurred during the *Daqu* fermentation process were monitored continuously (Figure 5.5). The core temperature

increased from 20°C to 52°C between stage 1 and stage 5, and thereafter decreased rapidly to ambient room temperature in stage 6. A gradual decrease of moisture content took place throughout the whole process, from 40 g water/100 g *Daqu* at the start to around 10 g water/100 g *Daqu* at the final stage. The changes in room temperature were less pronounced than those of the core of the *Daqu* blocks. With increasing temperature, the RH of incubation room dropped from approximately 100% to 20%.

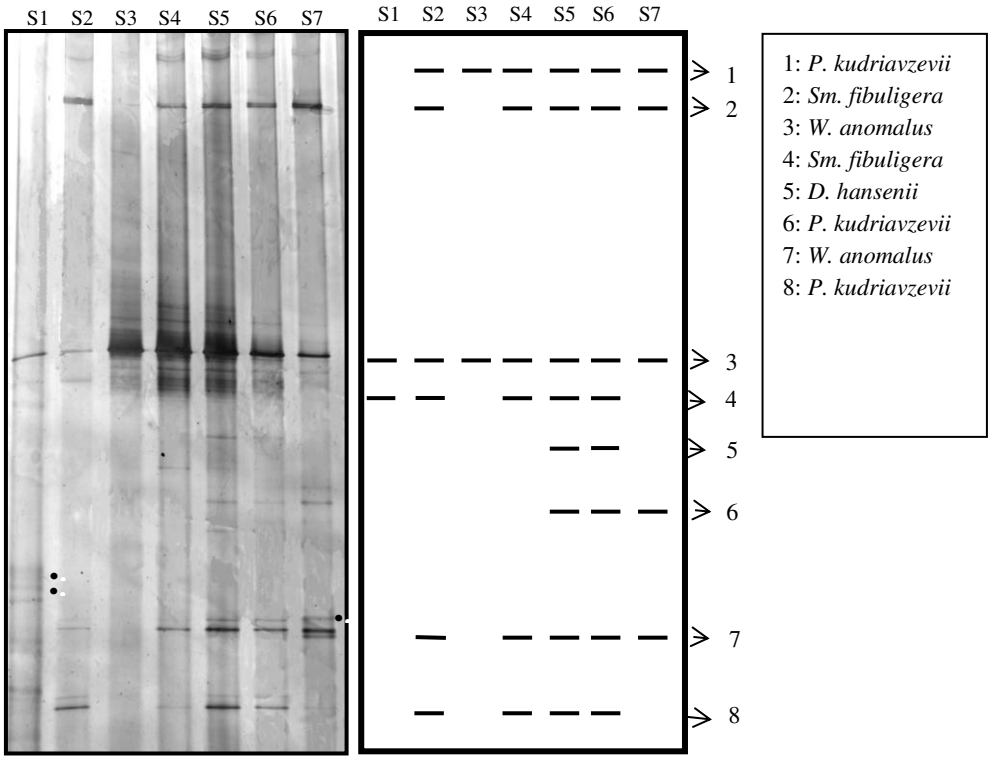

Figure 5.4 DGGE profiles (30-60% denaturant) representing 26S rRNA gene fragments of *Fen-Daqu* samples during fermentation (S1: Stage 1-*Woqu*; S2: Stage 2-*Shangmei*; S3: Stage 3-*Liangmei*; S4: Stage 4-*Chaohuo*; S5: Stage 5-*Dahuo*; S6: Stage 6-*Houhuo*; S7: Stage 7-*Yangqu*).The dotted bands were also excised but could not be identified because no amplicons were obtained.

Microbiota composition in relation to environmental variables

Canonical correspondence analysis (CCA) was carried out using abundant DGGE bands together with environmental variables. Species-environment correlations for both

axes were higher than 0.99 (canonical coefficients), suggesting that microbial diversity was strongly correlated with environmental factors. In the CCA triplot (Figure 5.6), the length of arrows indicated the relative importance of that environmental factor in explaining variation in microbial profiles, while the angle between the arrows (environmental factor - environmental factor or environmental factor-species) indicated the degree to which they were correlated. It shows that acidity, pH and surface temperature were the three most important environmental variables which could be correlated with the microbial diversity in different samples, since the lengths of these arrows are longer than those of others. Moisture content was the sole environmental factor that correlated with the microbial diversity at stages 3 and 7. Acidity correlated with the composition of the microbial community of stage 2, in which a relatively high abundance of *Ws. confusa*, *Ws. cibaria*, *B. cereus*, *B. pumilus* and *P. kudriavzevii* was found. Temperature and pH were found to be strongly correlated with the composition of the microbiota during the stages 4, 5 and 6.

5.4 Discussion

This study showed that species of the genus *Bacillus* are predominant during all stages of *Fen-Daqu* fermentation. By combining both culture-dependent and culture-independent data, stage 3 was identified as the stage with the highest diversity of *Bacillus* spp. This result is in agreement with the data of Yan et al. (2013b). In our study, seven *Bacillus* species, namely *B. anthracis*/*B. cereus*, *B. amyloliquefaciens*, *B. circulans*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilis*, were found during the whole *Daqu* fermentation process, whereas almost all these species were detected simultaneously at stage 3. Of these bacilli, only *B. licheniformis* and *B. subtilis* were found in the study of *Fen-Daqu* production by Li et al. (2013). This result emphasizes the importance of using two complementary methods because higher numbers of species could be obtained. *Bacillus* spp. can survive harsh environmental conditions such as drying (Deng et al., 2012). *Daqu* after stage 3 became dry due to evaporation, caused by the use of forced ventilation in order to control the incubation temperature during stage 3. This explains the decrease of both room temperature and core temperatures in Figure 5.5. As a result, the moisture content of *Daqu* decreased. Zhao et al. (2011) reported that bacilli have a better ability to survive

under low moisture conditions than other bacteria. Canonical correspondence analysis also revealed the significance of moisture content in correlation with the microbial communities at this stage (Figure 5.6).

The number of thermophilic bacteria and bacterial spores increased from 5.9 at stage 3 to 8.8 log CFU/g at stage 6 (Table 5.2), when the diversity of *Bacillus* spp. decreased. Correspondence analysis indicated that pH and temperature were the most important factors that correlated with the composition of the microbial community during the stages 4, 5 and 6. The core temperature during these three stages reached a maximum of about 52°C between stages 4 and 5 (Figure 5.5). Such high temperature is expected to have a selective effect, favouring thermotolerant, aerobic endospore-forming bacteria. Only *Bacillus* spp., such as *B. licheniformis* and *B. subtilis,* are able to grow between 50-60°C (Burgess et al., 2010). This may be the reason why members of the genus *Bacillus* spp. were dominating the microbial community during the last four stages. This could also explain why *B. licheniformis* and *B. subtilis* were encountered frequently in strong or sauce flavour *Daqu* (Wang et al., 2011b) in which fermentation temperatures are even higher than in *Fen-Daqu*.

LAB also play a role in the production of *Daqu* (Zheng et al., 2011), especially *Lactobacillus* spp. (Li et al., 2013). Acidity was recognized as the most significant factor that correlated with the composition of the microbial communities of stage 2 (Figure 5.6). The increase in acidity correlates with the occurrence of high numbers of LAB and fungi, especially *Lb. sanfranciscensis, Ws. cibaria, Ws. confusa, W. anomalus* and *P. kudriavzevii*.

The fungal community associated with *Fen-Daqu* fermentation was found to be less diverse than the bacterial one. A succession of yeast species during fermentation could not be observed. Li et al. (2013) reported the predominance of *P. kudriavzevii* in *Fen-Daqu*, however, in our study two other yeasts species (*Sm. fibuligera* and *W. anomalus*) were predominant during *Daqu* fermentation, since they occurred during almost the entire production process. *Sm. fibuligera* has been reported to degrade and assimilate raw starch as a carbon source (Chi et al., 2009) so it may contribute to the formation of fermentable carbohydrates for the subsequent alcoholic fermentation. *W. anomalus* and *P. kudriavzevii* are known as ester-producing yeasts and a higher abundance of these organisms correlate with an elevated concentration of esters in liquor (Li et al., 2012; Wu et al., 2012b).

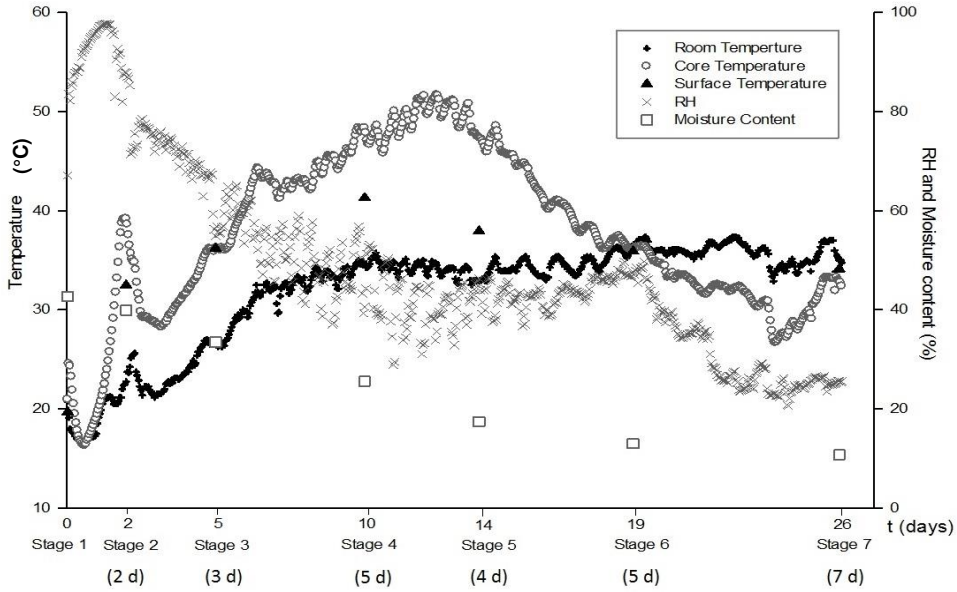

Figure 5.5 Changes in temperature, RH, and moisture content during fermentation of *Fen-Daqu*. (Stage 1: *Woqu*; Stage 2: *Shangmei*; Stage 3: *Liangmei*; Stage 4: *Chaohuo*; Stage 5: *Dahuo*; Stage 6: *Houhuo*; Stage 7: *Yangqu*).

Filamentous fungi are commonly used in solid state fermentations, because of their relatively high tolerance to low water activity and their production of hydrolytic enzymes (Rahardjo, 2005). The major moulds associated with *Daqu* fermentation can be categorized into two groups. The first group belongs to the family of Mucoraceae. These are known as strong amylase producers in amylolytic Asian fermentation starters (Rahardjo, 2005). Of these fungal species, *L. corymbifera* occurred during stage 5 and persisted until the end of the fermentation. The relatively high core temperature of the *Daqu* above 45°C during stages 4-5 allows only thermophilic or thermotolerant fungi to survive. This may be true for *L. corymbifera* that has been reported to grow at temperatures as high as 48-52°C (Weitzman et al., 1995), and may explain that it was detected during the last three stages of *Daqu* fermentation. The second group comprises *Aspergillus* spp., which are used in solid state fermentations and also are known to produce extracellular proteolytic and saccharolytic enzymes (Rahardjo, 2005).

In this study, both culture-dependent and culture-independent approaches were combined to analyse the dynamics of microbial communities during *Daqu* fermentation. Although both techniques may provide different results due to the use of various sampling methods, they showed sufficient overlap of information on the dominant groups of microbial communities. Only 6 bacterial species, i.e. *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis* and *Lb. plantarum* and 2 fungal species, i.e. *P. kudriavzevii* and *W. anomalus* could be detected by both approaches. By the culture independent method, no bands were identified as filamentous fungi. We assume that the absence of bands for filamentous fungi in our DGGE gel is mainly caused either by (1) inadequate DNA extraction of filamentous fungi from the complex food matrix of *Daqu*, or (ii) by the presence of high concentrations of competing DNA, such as that from yeasts in *Daqu*. These results reinforce the necessity of employing both culture-dependent and culture-independent approaches to uncover the microbial diversity of complex microbial ecosystems, such as *Daqu*.

Li et al. (2013) applied cloning methods to analyse the microbial communities during the production of *Fen-Daqu*, and they reported the predominance of lactobacilli and *P. kudriavzevii* in *Daqu*. However, our study showed the predominance of different groups of microorganisms at different stages of the fermentation. In conclusion, a succession in relative abundance of fungi, LAB and *Bacillus* spp. was observed during the *Daqu* fermentation process (Figure 5.1), which can be linked to changes in environmental conditions such as pH, temperature, acidity and moisture content. Better knowledge of microbial succession driven by environmental changes may facilitate long-term technological developments and innovation that will benefit the liquor and vinegar industry.

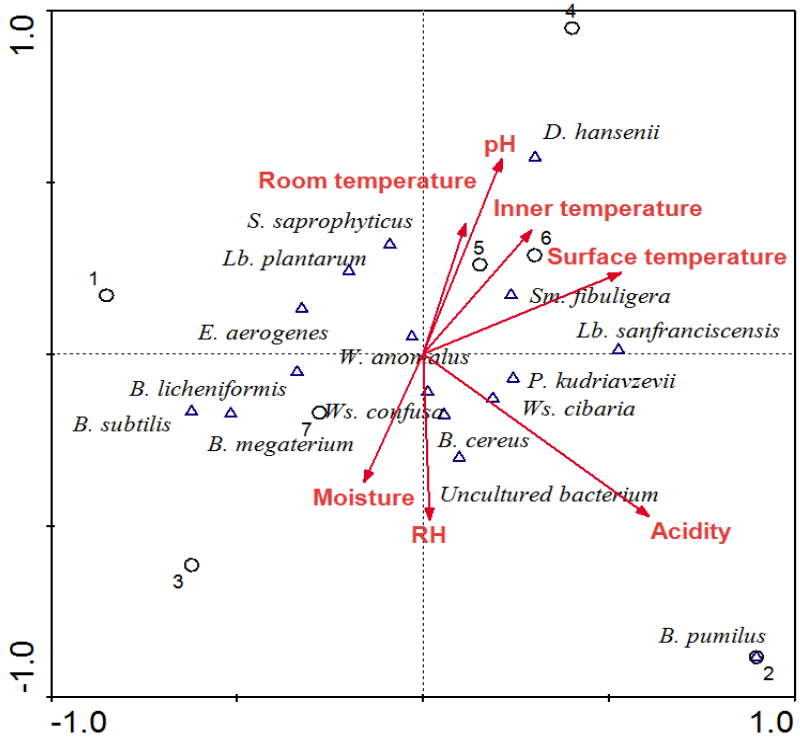

Figure 5.6 Canonical correspondence analysis (CCA) of DGGE community profiles from *Daqu* samples from the 7 stages. Numbers refer to the different stages, arrows refer to different environmental factors, and triangles refer to the individual abundant microbial species.

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Chapter 6

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

Abstract

Daqu is a traditional fermentation starter for the production of Chinese liquor 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 pentosaceu*s, *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. *Sm. 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*, *Sm. fibuligera*, *B. licheniformis*, *W. anomalus* and lactic acid bacteria in the production of light-flavour liquor (Fen-liquor).

6.1 Introduction

Chinese liquor 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*) followed by distillation (Zheng et al., 2011). Chinese liquor 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 Chinese liquor and vinegar are of microbial origin and produced during fermentation (Li et al., 2014a; Zheng et al., 2014). *Daqu* 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; Zheng et al., 2014). However, not much is known about the specific contributions of these microorganisms to the composition of Chinese liquor or vinegar. 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 et al., 2014b). Therefore, we performed an extensive screening exercise with the objective to select candidate species for use in starter cultures.

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

6.2 Materials and Methods

Screening of bacteria and yeast isolates for sorghum fermentation

(1) Cultivation of strains

A total of 161 microbial cultures, comprising 72 isolates of *B. licheniformis*, 46 isolates of *Sm. fibuligera*, 9 isolates of *P. kudriavzevii*, 26 isolates of *W. anomalus*, 1 isolate of *Saccharomyces cerevisiae*, and 7 isolates of LAB (*Ped. 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 (Oxoid CM035), and LAB were grown on MRSA (Oxoid CM0361). All stocks were stored in 30% glycerol at -80°C.

(2) Preparation of inoculum

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

(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.

Table 6.1 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

Note: B = *Bacillus licheniformis*; P.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.

(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. A crude enzyme solution was prepared according to the methods of Ali (1989) and Srivastava.(1986) The crude enzyme was obtained by centrifugation at 10,000 × *g* for 10 min at 4°C. Each time the obtained supernatant was used to assay for amyloglucosidase and α-amylase activities, respectively.

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.

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.

(5) Alcohol tolerance test

All strains were tested for their ethanol tolerance by using the spot test according to Kim.(2011a) 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.

Fermentation tests

(1) Preparation of inoculum

Based on the starch degradation and alcohol tolerance screening results using pure cultures of bacterial and yeast species isolated from *Fen-Daqu*, seven strains representing seven species were selected for lab-scale fermentation as shown in Table 6.1. Each strain (*B. licheniformis* 128, *Ped. pentosaceu*s 6, *Lb. plantarum* 1, *P. kudriavzevii* 12, *W. anomalus* 29, *S. cerevisiae* 1, and *Sm. fibuligera* 4) 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 2,000 × *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) Preparation of sorghum mixture

Aliquots of sorghum crude powder (100 g; obtained from Xinghuacun Fenjiu 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.

(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 6.1. 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 6.1). 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 6.1. 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-liquor (see materials and methods) was followed. One control fermentation with *Fen-Daqu* was used in this study.

Physicochemical analyses

(1) pH measurement

The pH of the fermentations was measured with a pin electrode and a pH meter inserted directly into each sample suspension (1g/10ml). Three independent measurements were done on each sample.

(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.

Chemical analysis

(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 2,500 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) NMR

The aqueous extracts for NMR measurements were prepared as reported previously.(Wu et al., 2009a) 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, 7,184 Hz; and time domain size, 32 k. The compounds were identified and quantified with Chenomx software (version 5.0; Chenomx, Edmonton, Canada) with reference to the internal standard TSP.

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 analysed by PCA using AMIX software (version 3.7.10;

BrukerBioSpin, Rheinstetten, Germany). Before performing the PCA, the NMR spectra (δ=0.70-9.20) were segmented into 0.04-ppm bins. The water region (δ=4.4-5.3) and imidazole regions (δ=7.35-7.50 and δ=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.

6.3 Results

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 *Ped. pentosaceus* and *Lb. plantarum* showed activities as high as 0.1-0.3 U/g (dw) (see Figure 6.1). *Ped. pentosaceus* 6 (P.p-6) and *Lb. 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, *Sm. fibuligera* had generally a higher starch degrading activity than the other 2 species (*P. kudriavzevii* and *W. anomalus*, see Figure 6.2). *W. anomalus* (W-29) showed high starch degrading ability (D_h/D_c: 1.4) and high α-amylase activity (0.144 U/g). Although *P. kudriavzevii* 12 did not produce α-amylase and amyloglucosidase, it showed starch degrading ability, with a D_h/D_c value of 1.4.

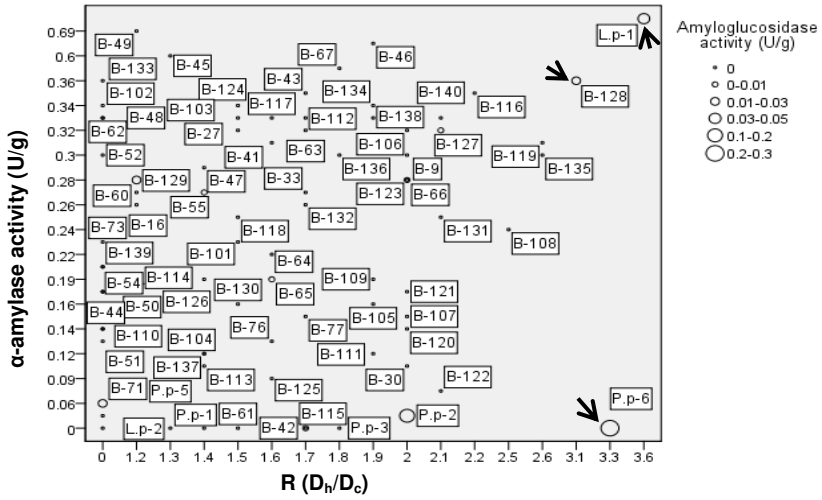

Figure 6.1 Bubble plot diagram of starch degradation (X-axis), α-amylase activity (Y-axis) and amyloglucosidase activity (bubble size, see legend top right) of bacterial strains. Note: B = *Bacillus licheniformis*; P.p = *Pediococcus pentosaceus*; L.p = *Lactobacillus plantarum*; D_h = The diameter of halo; D_c= the diameter of colony. Arrows indicate the selected strains.

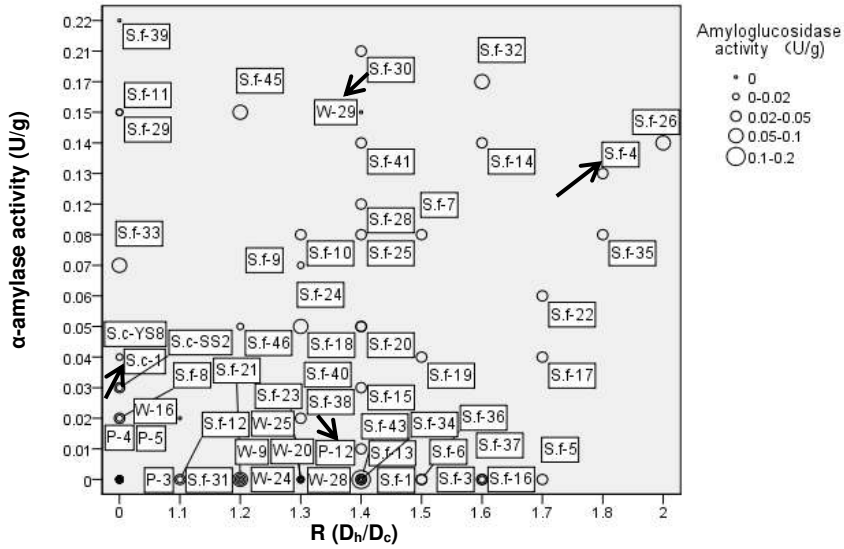

Figure 6.2 Bubble plot diagram of starch degradation of (X-axis), α-amylase activity (Y-axis) and amyloglucosidase activity (bubble size, see legend top right) yeast strains.

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.

More than 90% of the *Sm. fibuligera* strains were able to produce amyloglucosidase and α-amylase. The highest starch degrading yeast strain was *Sm. fibuligera* strain S.f-26. This strain not only had the highest ratio of 2.0, but also 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. By taking into account the α-amylase activity, amyloglucosidase activity, and the D_h/D_c-values, *S. cerevisiae* 1 showed the highest starch degradation ability. 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).

Table 6.2 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)	-	-	-
Ped. pentosaceus (2,6)	+	+	+
Ped. pentosaceus (5)	+	+	-
Ped. pentosaceus (1,3)	+	-	-
Lb. plantarum (2)	+	+	v
Lb. 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

Screening bacterial and yeast isolates for ethanol tolerance

The ethanol tolerance of bacterial and yeast strains is shown in Tables 6.2 and 6.3, respectively. Of the bacteria, *Ped. pentosaceus* 2 and 6 (P.p-2 and P.p-6) were able to grow up to 12% (v/v) alcohol, whereas *Lb. 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% alcohol, and the other strains showed variable tolerances of 4% and 8% alcohol (Table 6.3). 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 12% alcohol. Half of the *Sm. 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.

Table 6.3 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)	+	-	-
Sm. fibuligera (4,9)	+	+	+
Sm. fibuligera (6,43)	+	+	v
Sm. fibuligera (1,5,8,10,11,13,17,22,25,31,33,36,41,42,44)	+	+	-
Sm. fibuligera (16,28,34,37,38)	+	w	-
Sm. fibuligera (27)	+	v	-
Sm. fibuligera (2,3,7,12,14,15,18,19,20,21,23,24,26,29,30,32,35,40,45,46)	+	-	-
Sm. fibuligera (39)	v	-	-

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

Design of fermentation trials by mixing pure bacterial and yeast cultures

First, high starch degrading ability was used as criteria to select candidate bacterial and yeast strains for fermentation trails. Next, the ethanol tolerance of the selected strains

was compared. *B. licheniformis* 128 and *Ped. pentosaceus* 6 were selected based on the above mentioned criteria. Although strain *Lb. plantarum* 1 had a low ethanol tolerance, this species is frequently encountered in liquor fermentations (Li et al., 2011); therefore, we also included this strain in the fermentation trials. *W. anomalus* 29, *P. kudriavzevii* 12 and *S. cerevisiae* 1 were selected for their high starch degradation ability; *Sm. fibuligera* 4 and *Sm. fibuligera* 26 were similar in starch degradation ability, *Sm. fibuligera* 4 instead of *Sm. fibuligera* 26 was selected for its high alcohol tolerance.

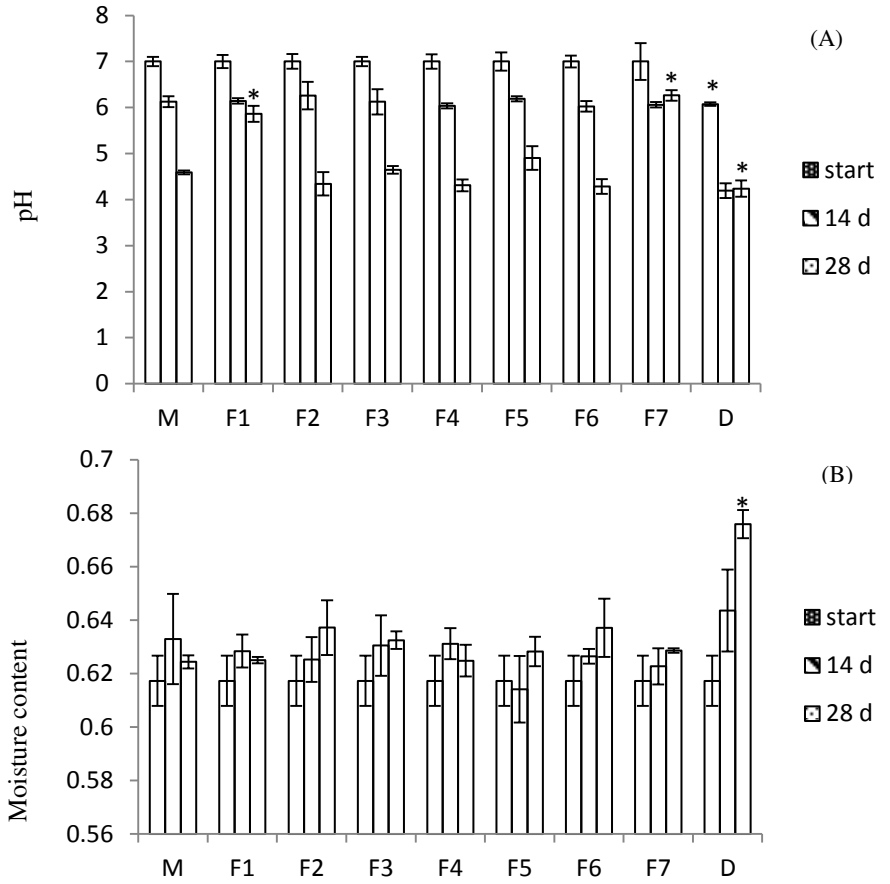

Figure 6.3 Changes of pH and moisture content in different fermentation trials at different stages of fermentation. The bars correspond to the estimated average levels of pH (A), and moisture content (B). The error bars indicate standard errors of the mean. * indicates the pH value or moisture content of the sample is significantly different from that of sample M.

Fermentation tests

(1) 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 (Figure 6.3A). 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.

Figure 6.3B 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.

(2) 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 (Figure 6.4). 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 scores plots in the different fermentation trials, loading plots of PC1 were generated (Figure 6.4I-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 *Ped. pentosaceus*), whereas opposite trend at lower acetate and higher lactate level were detected in fermentation 2 (without *Sm. fibuligera*) and 4 (without *P. kudriavzevii*) when compared to the levels in fermentation M. Figure 6.4N shows that the concentrations of lactate and acetate were all higher in fermentation 6 (without *Lb. 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 (Figure 6.4J). Ethanol was detected in higher concentration in fermentation 6, 7 and D (Figure 6.4N, O and P), with significant higher level in fermentation D. Figure 6.4P shows the differentiation of fermentation M and fermentation D (with *Daqu*). It shows that the concentrations of lactate, butyrate and acetate were higher in fermentation M.

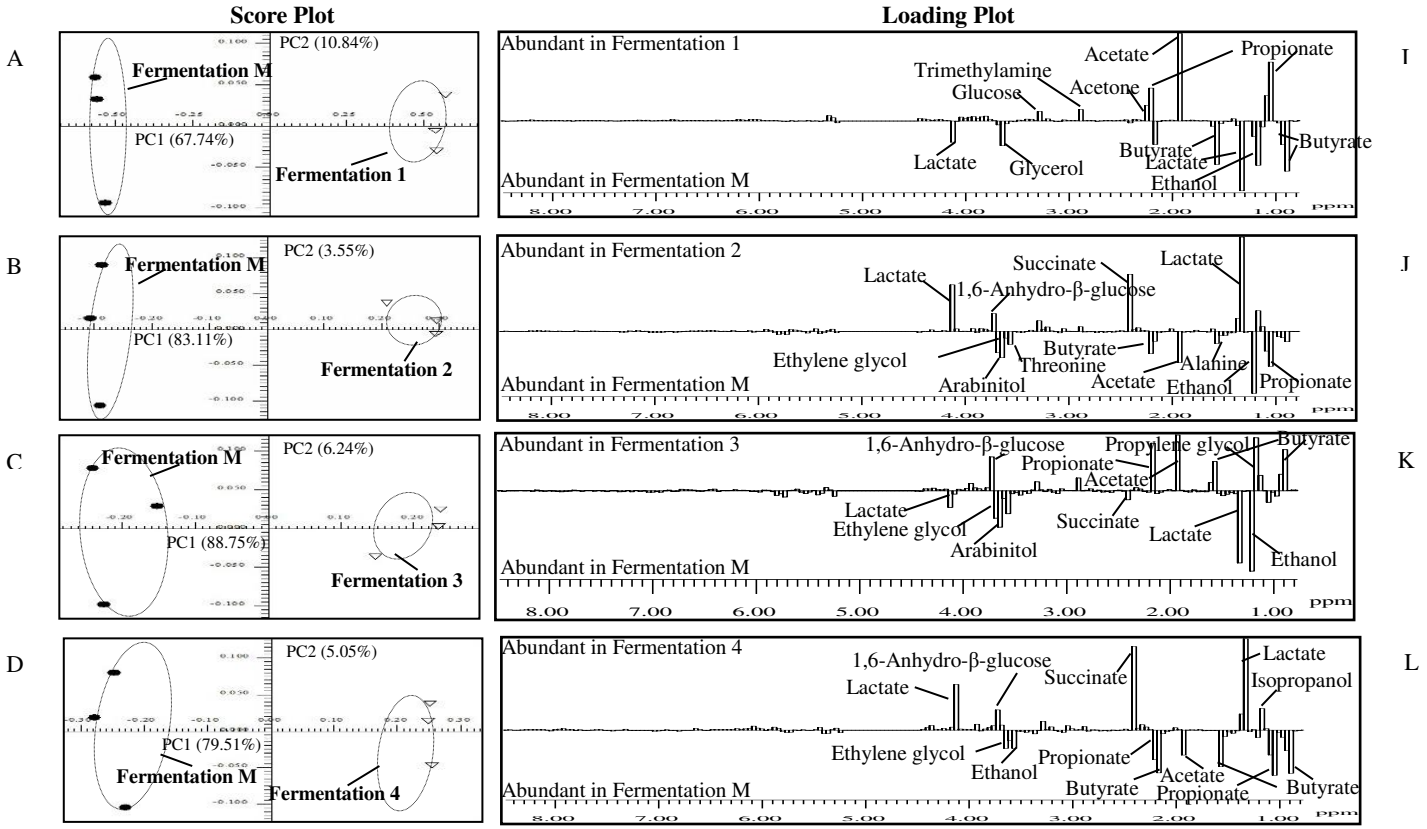

Figure 6.4 PCA scores (A-H) and loading (I-O) plots derived from the ^{1}H NMR spectra demonstrating significant statistical changes of metabolites in different fermentation samples.

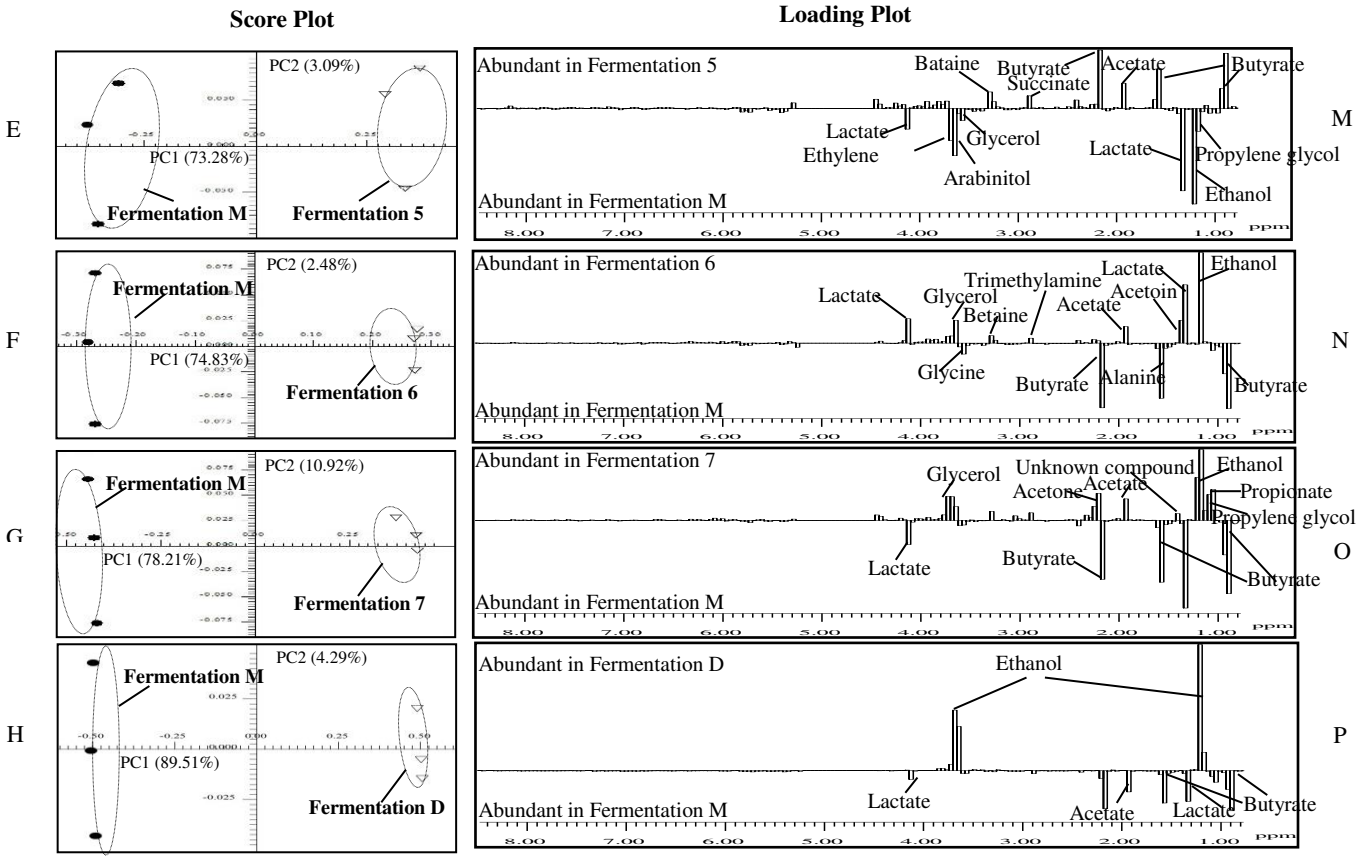

Figure 6.4 PCA scores (A-H) and loading (I-O) plots derived from the ^{1}H NMR spectra demonstrating significant statistical changes of metabolites in different fermentation samples (continued).

6.4 Discussion

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., 2012a; 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) (Figure 6.1). Some *Lb. plantarum* and *Ped. pentosaceus* strains (L.p-1 and P.p-6) showed higher starch degrading activities than *B. licheniformi*s. Generally, *B. licheniformis*, *Lb. plantarum*, and *Ped. 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 *Lb. plantarum* strain (L.p-2), and two *Ped. 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-fibres and structured filamentous growth when exposed to ethanol stress (Torres et al., 2005). *Lb. plantarum* and *Ped. pentosaceus* strains are known to be ethanol tolerant (Liu et al., 2009) due to solvent induced changes in the membrane lipid composition.

Sm. 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 *Sm. fibuligera* strains tested showed good starch degrading abilities (Figure 6.2).

Contributions of selected strains during alcoholic fermentation

(1) *Saccharomyces cerevisiae*

Saccharomyces 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 (Figure 6.4I 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 (Figure 6.3A). Two factors could lead to this change: a) alcoholic fermentation driven by *S. cerevisiae* is associated with the production of organic acids such as citric acids (Acourene et al., 2012); b) *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).

(2) *Saccharomycopsis fibuligera*

Saccharomycopsis 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 *Sm. 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 hydrolysed to glucose. *Sm. fibuligera* secretes almost exclusively α-amylase and glucoamylase (Ismaya et al., 2012), and this explains the high α-amylase activity of most tested *Sm. fibuligera* strains (Figure 6.2). Although *Sm. fibuligera* can produce a large amount of amylases that hydrolyse 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 *Sm.*

fibuligera could increase the production of ethanol, which is suggested by the higher ethanol concentration found in fermentation M than in fermentation 2 (without *Sm. fibuligera*). This effect was observed in other studies as well (Chen et al., 2010; Knox et al., 2004). In addition, the concentration of lactate was higher when *Sm. fibuligera* was not present in the fermentation. The accumulation of lactate suggests that *Sm. fibuligera* stimulates the conversion of lactate to other compounds, such as ethyl lactate. This compound might be abundant in fermentation M. Moreover, *Sm. 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 Figure 6.4J, 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 *Sm. fibuligera*, especially its high acid proteinase activity (Chi et al., 2009). Several studies have reported that *Sm. 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 alcoholic fermentation industry, because it could hydrolyse 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*-liquor as described by Xiong et al.(2005a).

(3) *Wickerhamomyces anomalus* and *Pichia kudriavzevii*

Wickerhamomyces anomalus is regarded as an important aroma producer that is present in a wide range of fermented products (Nyanga et al., 2013; Soka et al., 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 Chinese liquor 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*) (Figure 6.4K). 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* is associated with mycotoxin production (Moss, 2002).

Pichia kudriavzevii is another good ethanol producer (Yuangsaard et al., 2013), which is confirmed by our results (Figure 6.4L) that showing a higher ethanol concentration in fermentation M if compared to fermentation 4 (without *P. kudriavzevii*).

(4) *Bacillus licheniformis*

Bacillus 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, Figure 6.1). Starch comprises 65-81% of the total weight of sorghum grains (Cao et al., 2010b). 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., 2013a), 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 liquors such as *Fen*-liquor (Yan et al., 2013a). Ethyl lactate is mainly produced via esterifying enzymes secreted by other microorganisms, such as butyric acid bacteria, during the fermentation of Chinese liquor (Sun, 2014). Therefore, *B. licheniformis* most likely makes an important contribution to the formation of flavours and aromas of Chinese liquor 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*).

(5) *Lactobacillus plantarum* and *Pediococcus pentosaceus*

The two LAB species *Lb. plantarum* and *Ped. pentosaceus* are frequently encountered in *Daqu* and fermented grains (Li et al., 2013; Zheng et al., 2012; Zheng et al., 2014). Amylolytic activity of *Lb. 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 Figure 6.1). Our results also showed its proteolytic activity during fermentation, as amino acids, such as glycine and alanine, were produced (Figure 6.4N). 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 Chinese liquor, especially light-flavour liquors, such as *Fen*-liquor, as this type of liquor has a pure and sweet taste (Xiong, 2005a). The PCA analysis of metabolite profiles of fermentations M and 6 revealed one unexpected result: the concentration of lactate was higher when *Lb. plantarum* was absent. One possible

reason for this is that *Lb. plantarum* could convert lactate to acetate under limited oxygen conditions (Goffin et al., 2004; Liu, 2003).

Ped. 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 (Figure 6.3). This indicates that *Ped. 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 *Lb. plantarum*, *Ped. 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 (Figure 6.4H). 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*, *Sm. fibuligera*, *W. anomalus, P. kudriavzevii*, *B. licheniformis*, *Ped. pentosaceus*, and *Lb. plantarum*) (Figure 6.4P). *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. cevervisiae* resulting in higher ethanol yields. However, fermentation M tends to produce more acid, especially lactate and acetate, instead of ethanol (Figure 6.4P). The reason for this observation could be the high proportion of *Lb. plantarum* and *Ped. pentosaceus* added to the mixtures. As described previously, about 10^7 CFU/g *Lb. plantarum* and *Ped. 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 (Figure 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, *Sm. fibuligera* 4, *W. anomalus 29*, *P. kudriavzevii* 12, *B. licheniformis* 128, *Ped. pentosaceus* 6, and *Lb. plantarum* 1 strains may be regarded as important for *Daqu*. However, the behaviour of *Daqu* could not yet be simulated completely. Further study involving the additional effect of filamentous fungi will be required.

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Supplementary data

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

Table S1 Presumptive metabolites and their contents (micromoles per gram of fermented grain) in samples during the alcoholic fermentation M, and 1-7 and D

Metabolites	Start	M		F1		F2		F3		F4		F5		F6		F7		D	
	0d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d
1,6-Anhydro-β-glucose	0.67	nd	nd	nd	nd	0.42	0.36	0.56	0.65	0.34	0.88	0.44	nd	nd	nd	nd	nd	0.62	nd
4-Aminobutyrate	0.98	1.57	0.35	1.49	1.86	0.57	0.72	1.71	0.91	1.34	0.98	0.44	0.18	0.43	0.48	1.45	1.73	1.74	1.78
3-Hydroxyisovalerate	0.04	0.15	0.15	0.03	0.05	0.03	0.05	0.01	0.01	nd	nd	0.02	0.04	0.04	0.02	0.01	0.00	0.16	0.81
4-Hydroxybutyrate	nd	0.21	0.24	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.14	0.29
2-Phosphoglycerate	0.24	0.21	0.27	0.68	0.95	0.23	0.84	0.08	nd	0.27	1.13	0.21	0.14	nd	nd	nd	nd	0.58	2.00
Acetate	8.87	14.61	16.79	14.06	25.00	0.57	12.82	15.64	25.36	15.61	11.28	14.86	26.84	15.76	30.81	15.83	20.15	6.94	7.94
Acetoacetate	0.27	0.31	0.35	0.14	0.39	0.11	0.07	0.01	0.01	0.06	0.06	0.01	0.09	0.02	nd	0.03	0.12	0.01	0.15
Acetone	0.41	2.95	0.05	2.17	1.24	1.84	0.12	1.87	0.08	2.13	0.07	1.46	0.19	1.14	0.32	2.32	1.58	0.65	1.76
Adenine	0.28	0.27	0.28	nd	nd	0.23	0.31	0.04	0.01	0.06	0.16	nd	nd	0.07	0.01	0.04	0.07	0.03	nd
Adipate	nd	nd	nd	nd	nd	0.14	0.22	nd	nd	0.03	1.26	nd	nd	nd	nd	nd	nd	nd	nd
Alanine	0.42	0.71	1.30	0.61	1.25	0.23	0.27	0.73	0.99	0.45	0.93	0.14	1.10	0.18	0.38	0.41	1.10	2.38	3.33
Arabinitol	0.42	0.56	0.44	0.23	0.22	0.32	nd	nd	nd	nd	nd	0.10	0.21	nd	nd	nd	nd	4.29	0.10
Arginine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.58	nd
Ascorbate	nd	0.50	0.12	0.13	0.13	nd	nd	0.10	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.02	0.64
Aspartate	nd	nd	nd	nd	nd	nd	nd	0.05	nd	nd	nd	nd	nd	nd	nd	0.08	nd	nd	nd
Betaine	1.43	0.85	0.29	0.68	0.56	0.73	0.69	0.82	0.59	0.63	0.53	0.64	1.25	0.79	0.91	0.73	0.77	1.83	1.19
Butyrate	0.06	0.66	27.72	1.11	2.14	0.24	14.15	0.68	31.57	0.57	15.42	0.07	32.47	0.73	15.42	0.60	1.91	0.88	0.82
Carnitine	0.34	0.20	0.13	0.16	0.12	0.09	0.21	0.19	0.06	0.15	0.17	0.10	0.07	0.11	0.11	0.16	0.11	2.17	1.94
Choline	0.40	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.02	0.06
Creatine	0.28	0.01	nd	nd	nd	nd	nd	nd	0.22	nd	nd	nd	nd	nd	nd	nd	nd	0.15	0.25
Creatinine	0.32	nd	nd	nd	nd	nd	nd	nd	0.11	nd	nd	nd	nd	nd	nd	nd	nd	0.18	0.06
Cysteine	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.03	0.14	0.03	0.05	nd	nd	nd	nd	0.05	0.22
Ethanol	13.32	41.05	36.40	34.97	12.45	5.07	19.58	43.17	21.58	23.29	26.51	4.51	7.11	5.85	44.62	32.63	43.24	98.31	153.80

Table S1 Presumptive metabolites and their contents (micromoles per gram of fermented grain) in samples during the alcoholic fermentation M, and 1-7 and D (continued)

Metabolites	Start	M		F1		F2		F3		F4		F5		F6		F7		D	
	0d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d
Ethylene glycol	0.36	0.12	1.45	0.29	0.25	0.09	0.05	0.19	0.03	0.24	0.03	0.24	0.29	1.23	1.33	1.28	1.47	1.29	1.28
Fructose	1.58	0.11	nd	nd	nd	nd	nd	nd	nd	0.40	nd	nd	0.18	nd	nd	nd	nd	1.66	1.44
Galactose	0.63	0.26	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.13	0.69
Galactonate	0.34	0.14	0.04	0.05	0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.74	0.04
Glucose	2.23	nd	nd	0.14	0.24	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	9.28	16.56
Glucitol	0.87	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.12	0.19	nd	nd	nd	nd	2.62	3.32
Glutamate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.48	1.97
Glycerol	5.38	1.50	2.23	1.40	0.76	1.46	1.98	1.52	1.90	1.50	1.75	1.47	0.70	1.55	3.83	1.55	3.03	18.36	31.10
Glycine	0.54	0.61	1.58	0.90	0.82	0.21	0.35	0.27	0.31	0.15	0.45	0.34	0.27	0.08	0.31	0.43	0.99	1.18	1.68
Histamine	0.28	0.22	0.22	0.07	0.06	nd	nd	0.10	0.05	nd	0.01	0.34	0.11	0.04	0.03	0.22	0.31	0.62	0.61
Imidazole	4.25	4.20	3.87	4.21	4.23	4.23	4.32	4.06	7.95	4.40	4.16	4.27	4.18	4.19	4.07	4.31	4.15	3.42	2.54
Isoleucine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.61	0.68
Isopropanol	0.29	0.30	0.17	0.26	1.96	nd	nd	nd	nd	0.23	1.22	0.04	0.88	0.15	0.72	0.13	0.92	0.99	1.28
Lactate	0.32	0.44	17.25	0.38	0.30	0.77	28.54	0.48	7.32	0.23	23.31	0.13	5.64	0.26	33.70	0.26	0.12	9.84	6.09
Leucine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.90	1.46
Malate	0.18	nd	nd	nd	nd	0.12	nd	0.03	0.07	0.04	nd	nd	nd	nd	nd	nd	nd	0.09	0.10
Maltose	0.33	1.21	nd	1.19	nd	1.02	0.03	1.04	nd	1.08	nd	0.03	nd	1.32	nd	1.09	nd	0.72	0.06
Methanol	0.12	0.28	0.41	0.30	0.56	0.29	0.78	0.33	0.33	0.23	0.23	0.25	0.32	0.62	0.43	0.26	0.34	0.09	0.84
Oxypurinol	0.29	0.65	1.72	0.49	0.44	0.31	0.79	0.31	0.25	0.82	0.99	0.86	0.81	0.79	0.99	0.22	0.21	0.23	0.19
Proline	0.65	1.24	0.97	0.92	0.97	nd	1.23	nd	nd	nd	nd	nd	nd	nd	nd	1.24	1.28	4.51	6.09
Propionate	1.05	1.06	9.11	1.17	19.20	1.10	1.21	1.18	13.81	3.70	1.19	1.13	6.34	0.03	0.03	1.31	19.80	0.21	0.43

Table S1 Presumptive metabolites and their contents (micromoles per gram of fermented grain) in samples during the alcoholic fermentation M, and 1-7 and D (continued)

Metabolites	**Start**	**M**		**F1**		**F2**		**F3**		**F4**		**F5**		**F6**		**F7**		**D**	
	0d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d
Propylene glycol	0.60	0.50	0.68	0.24	0.26	nd	0.04	nd	1.17	nd	0.06	nd	nd	nd	nd	0.46	1.32	0.66	1.13
Pyruvate	0.10	0.04	0.12	0.17	0.17	0.10	0.03	0.01	0.03	0.02	0.03	0.02	0.03	0.03	nd	nd	nd	0.34	0.43
Ribose	1.61	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.56	5.62
Serine	0.01	nd	0.01	0.08	0.09	nd	nd	nd	nd	0.10	0.06	0.03	0.10	nd	nd	nd	nd	0.18	1.71
Succinate	2.12	8.22	1.23	6.64	0.24	4.81	5.88	9.00	0.34	5.22	6.07	3.45	3.04	4.92	1.59	7.48	0.050	4.80	4.38
Sucrose	0.60	0.29	0.19	0.17	0.16	0.01	0.01	nd	nd	nd	0.02	nd	nd	nd	nd	0.01	0.03	0.07	0.50
Threonine	0.14	0.76	1.10	1.00	1.23	0.23	0.61	0.82	1.24	0.92	0.99	0.92	0.99	0.87	1.34	0.97	1.25	0.98	1.35
Trimethylamine	0.02	0.78	0.35	0.78	1.46	0.93	0.85	0.95	1.15	0.76	0.67	0.72	0.95	0.88	1.53	0.82	1.09	0.11	0.11
Urea	1.21	2.32	3.83	0.98	0.04	1.40	1.52	0.97	0.91	1.37	0.94	1.24	1.15	2.09	1.10	1.32	2.14	0.23	nd
Valine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.52	0.84

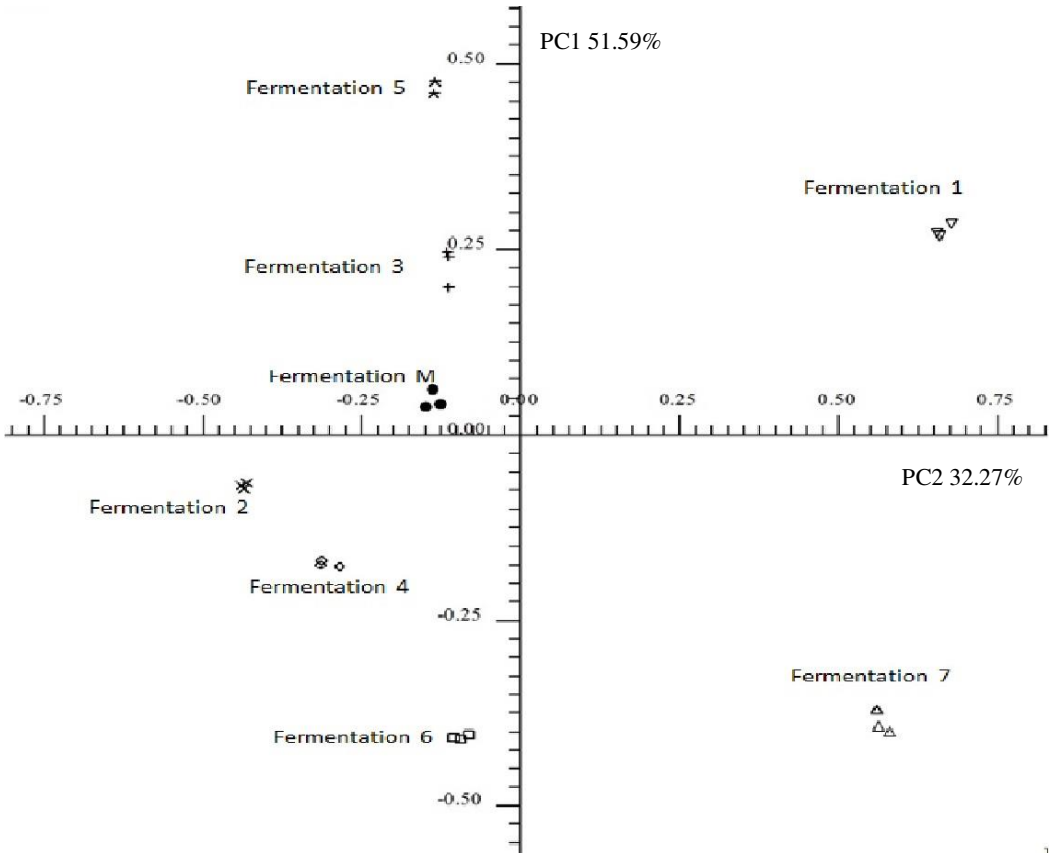

Figure S1 PCA score plot of triplicate samplings of different fermentation trails.

Chapter 7

General discussions and future perspectives

Chinese liquor is one of the best-known Chinese traditional fermented products. It is manufactured through a three-step process of which the first step is a solid-state fermentation of cereals initiated by the addition of a starter culture. The second step is the distillation of the fermented cereals followed by the third and final step: long term ripening of the distilled product (Tsao et al., 2010). The starter culture for this process (called *Daqu*) is also produced by a solid-state fermentation process. The temperature during the production process of the starter (*Daqu*) is variable and ranges from 25°C up to 65°C. The process is generally divided into three phases: a low-temperature incubation phase, a high-temperature phase, and a final aroma-generating phase (**Chapter 2**). The duration of each phase depends on the type of *Daqu* that is produced (Shen, 2001a).

Alcoholic fermentation starts with the mixing of milled and cooked grains (mainly sorghum) with hot water, and subsequently the addition of powdered *Daqu*. The mixture is then loaded into cellars fermenters (length ×width × height, 3.4 × 1.8 × 2.0 m) and solid-state fermentation is carried out at 28°C-32°C for 28-60 days under anaerobic conditions (Li et al., 2013).

The microbial composition of the *Daqu* directly impacts the flavour and microbial safety of the *Daqu*-fermented products (Huang et al., 2013). Although the microbial composition of *Daqu* depends on a combination of factors, including the choice of raw materials, climate, and production techniques, fermentation conditions such as temperature, moisture content, and oxygen availability are the most critical factors.

The studies described in this thesis focus on one of the most widely used *Daqu* types in Chinese liquor production. *Daqu* classification (sauce-flavour *Daqu*, strong-flavour *Daqu*, and light-flavour *Daqu*) and traditional production processes, as well as the influence of fermentation conditions on the quality of *Daqu* starters has been reviewed. The results presented in **Chapter 3** demonstrate that geographic location and fermentation temperature are the most important factors determining the microbial community structure of *Daqu*. **Chapters 4** and **5** deal with the microbial diversity at the surface and in core fractions of *Daqu* as well as microbial succession during the fermentation process. The relationship between environmental factors including acidity, moisture content, and temperature and the microbial composition at different fermentation stages was described in detail. Finally, the

most common groups of microorganisms in an representative low-temperature *Daqu* (*Fen-Daqu*) [*Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Bacillus licheniformis*, *Wickerhamomyces anomalus*, and lactic acid bacteria (LAB)] were identified as being involved in the main processes such as saccharification, protein degradation, alcohol production, and flavour generation during alcoholic fermentation, thus highlighting the importance of *Daqu* as a starter culture.

In the following sections, the most important results are discussed from an integrative perspective, and the advantages and disadvantages of different techniques for studying microbial diversity in food products are summarized and discussed. Future perspectives for research on *Daqu* production and *Daqu* driven alcoholic fermentations are outlined with focus on traditional fermented foods.

7.1 Characterization of *Daqu* fermentation activity

General information on the composition and processing of *Daqu* and its application in alcoholic fermentation processes were presented in **Chapter 2**. *Daqu* is a complex mixture of chemical compounds, microbes, and enzymes (Zheng et al., 2011). The composition of microbial communities in *Daqu* used by different liquor factories has traditionally been characterized based on culture-dependent or -independent approaches such as DGGE, PLFA, and cloning (Liu et al., 2012a; Zhang et al., 2014; Zheng et al., 2015). Although various *Daqu* types contain different microbial profiles, four major groups of microorganisms - *Bacillus* spp., LAB, non-*Saccharomyces* yeasts, and moulds - are present in all *Daqu* types. Among these organisms, *B. licheniformis* and *Sm. fibuligera* are most frequently isolated from *Daqu* and are present at all stages of fermentation (Liu et al., 2012a; Zhang et al., 2014; Zheng et al., 2015), consistent with the results of our study (**Chapter 2, 3,** and **5**). These species produce amylases that facilitate the conversion of starch into monomeric sugars and proteases that liberate peptides and amino acids (**Chapter 6**). In addition, *B. subtilis* and acetic acid bacteria are the predominant species in source-flavour *Daqu* (Wang et al., 2011b), in which the amino acids isoleucine, leucine, isovalerate and valine are the most important components; this may associated with the high protease activity of *Bacillus* spp. (Wu et al., 2009a).

LAB (primarily lactobacilli), which confer organoleptic properties to various fermented foods (Leroy et al., 2004; Sumby et al., 2014), have been detected in *Daqu* at concentrations of 4-6 log CFU/g and are associated with the rapid acidification of raw materials (sorghum) and the formation of specific aroma compounds found in Chinese liquor (*Baijiu*) and vinegar (**Chapter 6**). In addition, LAB in *Daqu* produces metabolites such as ethyl lactate, which is one of the main aromatic compounds in light-flavour Chinese liquor.

Non-*Saccharomyces* yeasts contribute significantly to the overall character of wine (Ciani et al., 2010; Fleet, 2008). The present study confirmed that some non-*Saccharomyces* species in *Daqu* such as *Sm. fibuligera* and *Pichia kudriavzevii* could enhance the ethanol production of *S. cerevisiae*, and species such as *W. anomalus* could increase the ester composition of the liquor, thereby conferring a more complex flavour. The moulds found in *Daqu* are thought to play a role in starch degradation as well as flavour generation during alcoholic fermentation of cereals. Mould growth is highly dependent on environmental conditions such as humidity, and ambient temperature (Brown et al., 2001). No moulds were present in all *Daqu* types, and moulds were therefore not characterised in our study. However, previous findings have indicated that moulds in *Daqu* are associated with starch degradation and the formation of esters and other volatile compounds (Ma et al., 2011; Zheng et al., 2011). Our current understanding of the role of moulds present in *Daqu* is fragmentary, and detailed studies are required to fill in the gaps and to obtain more insight into their role in the fermentation process.

Daqu is not only a source of fermenting microorganisms but also of hydrolytic microbial enzymes such as α-amylase and amyloglucosidase (**Chapter 6**). The majority of *Daqu*-isolated *B. licheniformis* and *Sm. fibuligera* strains exhibit these activities, which are essential for starch degradation. Therefore, microbial species in *Daqu* are involved in the saccharification of raw substrates such as barley and pea during *Daqu* processing and sorghum during alcoholic fermentation. Other enzyme activities, including those of proteinases, esterases, lipases, cellulases, hemicellulases, tannase, and pectinases were reported to be present in *Daqu* (Zhang et al., 2011d); however, their origin and functional significance need to be determined.

A mould strain (TR12) with high starch utilization under acidic conditions was isolated from *Luzhou Daqu* (Fang et al., 2007); using it as a starter culture for alcoholic fermentation improved liquor production and aroma (Zhang et al., 2009). A systematic investigation of *Daqu* composition including microbial community structure and activity, enzymatic profile, and chemical components, is a prerequisite for understanding traditional liquor production techniques and for selecting starter cultures that produce the beverages with specific flavour.

7.2 Safety evaluation of *Daqu*

Daqu contains a complex mixture of different live microorganisms. During its production, an environment that supports the required microbiota while suppressing undesirable growth must be created to ensure the quality and safety of *Daqu* and its products (Hai et al., 2014). Although pathogenic microorganisms can be introduced by workers, production tools, and livestock feed during *Daqu* production (Shu et al., 2010), these microorganisms can not lead to infections in consumers since they will be pasteurized under distillation. However, metabolites of contaminating microorganisms can influence the growth of functional microbiota, and therefore, such contaminated products may pose a safety risk, therefore the toxicological safety of *Daqu* must be monitored and controlled. To identify contaminating and potentially harmful microorganisms this study addressed the microbial diversity in different types of *Daqu* (**Chapter 2**) and its influence on the *Daqu* production process (**Chapters 3**, **4**, and **5**).

The presence of mycotoxin-producing fungi in *Daqu* is a potential risk factor for consumers of Chinese liquor and vinegar; several studies have identified mycotoxins and ethyl carbamate (EC) as potentially toxic factors (Fan et al., 2012a). Fungi such as *Aspergillus flavus* and *Penicillium nordicum* produce aflatoxin B_1 and ochratoxin respectively (Moss, 2002), which are two mycotoxins frequently found in beer, wine, and condiments such as soy sauce and vinegar (Cao et al., 2010c; Sun, 2009; Yang et al., 2013). Of the 59 samples that included beer, soy sauce, and vinegar, 58 contained $\leq$ 5 μg/kg aflatoxin B_1 and the remaining sample contained 8 μg/kg (Cao et al., 2010). In other studies, all of the tested sample contained ochratoxin at levels ranging from 0.14 to 1.10 μg/kg

(Chen et al., 2012; Sun, 2009). The National Standards of the People's Republic of China (GB2761-2011) has set the maximum level in grain-based food products at 3μg/kg for ochratoxin and 5 μg/kg for aflatoxin B_1, respectively (China State Bureau of Standards, 2011), while the European Parliament has set the maximum level in grain-derived foodstuff or wine at 2 μg/kg for both toxins (European Parliament, 2006). The concentrations of these two mycotoxins in various Chinese liquors and starter *Daqu* range from 0.1 to 4 μg/kg (Sun, 2009; Yang et al., 2013; Ye et al., 2013); higher mycotoxin content can pose a risk for consumers. It is therefore necessary to control their levels by minimizing contamination by mycotoxin-producing fungi. The present study found that four *Daqu* producers (9-H-S, 1-H-S, 7-L-N, and 2-H-S) may have issues related to mycotoxin contamination (**Chapters 1** and **2**); however, in each case, the actual presence of mycotoxins has not been confirmed and contamination levels need to be analysed before further action is taken.

Ethyl carbamate (EC) is a potentially carcinogenic compound frequently present in fermented foods, baked foods and alcoholic beverages (Zhao et al., 2013). EC is formed from various precursors such as hydrocyanic acid, urea, citrulline and N-carbamyl compounds (including carbamyl phosphate) by reacting with ethanol (Weber et al., 2009) under acidic conditions; the reaction is favoured by increasing temperature and acidic pH (Araque et al., 2009). Several lactic acid bacteria (LAB) including *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Leuconostoc citreum*, and *Lactobacillus pseudomesenteroides*, are involved in the production of these precursors (Patrignani et al., 2012; Qian, 2012; Zhao et al., 2013). Our analysis showed that three commercial *Daqu* samples (4-M-S-W, 7-L-N-BP, and 7-L-N-BP') contained one or two of these species. EC content was generally higher in high- than in low-temperature or medium temperature *Daqu* (2012b). In various local Chinese alcoholic beverages, EC levels were in the range of 88-241 μg/l (Wu et al., 2012a). Given that alcoholic beverages represent the most likely source of EC intake, several countries have set limits on acceptable levels of consumption: in the United States, the maximum level is 15 μg/l for table wines and 100 μg/l for fortified wines (Zhao et al., 2013), and in Canada, the limits are 30 μg/l for table wines, 100 μg/l for fortified wines, and 150 μg/l for distilled spirits (Battaglia et al., 1990). The EC content in Chinese liquor are high relative to these standards; as such, EC has recently received considerable attention

due to the challenges faced by the liquor industry (Fan et al., 2012b; Zhao et al., 2013). Reducing EC content in *Daqu* is the most important measure for controlling EC level in the final liquor. EC content was lower in High temperature *Daqu* than in Medium or low temperature *Daqu* (Fan et al., 2012b), which may be explained by the high levels of LAB present under the latter conditions. The findings of this study can help liquor factories track potentially harmful metabolites and microorganisms in their products that pose the risk of mycotoxin and toxin contamination.

7.3 Contribution of bacteria and yeasts isolated from *Fen-Daqu* to alcoholic fermentation

The production of *Fen-Daqu* follows a strict temperature regimen (**Chapters 2** and **5**). *B. licheniformis*, *Sm. fibuligera*, *W. anomalus*, *P. kudriavzevii, Lichtheimia corymbifera*, and LAB are the predominant microbial species found in *Fen-Daqu* and are active at different stages of alcoholic fermentation (**Chapter 6**).

S. cerevisiae is the most important species in the fermentation process owing to its ability to convert glucose, sucrose, maltose, and galactose into ethanol. Although *S. cerevisiae* was not predominant in the starter (*Daqu*), it grew rapidly and became predominant during alcoholic fermentation owing to its competitive growth in the presence of fermentable sugars and high ethanol tolerance. Similar results were obtained in a previous study (2012). In this study, the *S. cerevisiae* strain produced the highest concentration of ethanol than other yeast species **(Chapter 6)**.

B. licheniformis and *Sm. fibuligera* are predicted to perform saccharification of sorghum starch to release soluble sugar monomers, which can be converted to alcohol by yeast fermentation. High levels of amyloglucosidase and α-amylase production by *B. licheniformis* and *Sm. fibuligera* (**Chapter 6**) confirmed the saccharification activity of these two species. While important for providing fermentable sugars, these also affect the flavour of the products since *Sm. fibuligera* secretes an acidic proteinase (Chi et al., 2009) that stimulates the release of amino acids such as alanine and threonine (**Chapter 6**), which react with glucose to generate volatile compounds under acidic conditions during distillation by the Maillard reaction and confers a sweet and fruity odour to Chinese liquor.

Additionally, metabolites from microorganisms contribute to the main flavour of *Daqu*-derived products (Zhang et al., 2011b). The major aroma-forming compounds in *Fen*-liquor are ethyl acetate and ethyl lactate (Yan et al., 2013a); the former is generated by *B. licheniformis*, indicating that this species may contribute to the palatability of the final product.

Two other non-*Saccharomyces* yeast species that were examined, *W. anomalus* and *P. kudriavzevii*, had different effects on ethanol production and flavour development. *P. kudriavzevii* produced ethyl acetate in *Fen-Daqu*-derived products such as *Fen*-liquor (**Chapter 6**), which is also produced from acetate by *W. anomalus* via esterase activity. Ethyl acetate has a distinctly sweet and fruity aroma that significantly improves the final taste of *Fen*-liquor; however, it can also have a disagreeable flavour at concentrations above the threshold level of 0.02 g/l. Therefore, the esterase activity of *W. anomalus* must be carefully regulated in order to avoid leaving traces of this flavour in the products.

LAB are the second largest group of bacteria in *Fen-Daqu* at around 5-6 log CFU/g (**Chapter 5**). *Lb. plantarum* and *Ped. pentosaceus* were the most abundant LAB in *Fen-Daqu*, and were present throughout the entire *Daqu* production process (**Chapter 5**) and during alcoholic fermentation of *Fen*-liquor (Li et al., 2013). Both species are predicted to produce flavour precursors (mainly lactic acid), while only *Lb. plantarum* converts lactic acid to ethyl lactate (**Chapter 6**). Some strains of *Lb. plantarum* have proteolytic activity and release amino acids such as arginine, cysteine, glutamate, and isoleucine (Pastink et al., 2009), which may also contribute to the complex aroma of liquor or vinegar.

7.4 Effect of environmental factors on microbial diversity

Biomarkers have been applied to food, clinical, and environmental sciences with the aim of fast identification of food safety, disease manifestation and environmental pollution (Fadda et al., 2010; Ptolemy et al., 2010; Wagner et al., 2007). The ideal biomarker provides a rapid and specific readout of environmental conditions or product characteristics. The identification and characterization of biomarkers that distinguish *Daqu* from different locations, factories, or products produced at different fermentation temperatures (i.e., attribute-specific biomarkers) is essential not only for verifying the authenticity of *Daqu*

but can also provide criteria for evaluating new processing methods or *Daqu* types. The present microbiological analyses demonstrated a higher degree of heat sensitivity of fungal communities as compared to bacterial communities present in *Daqu* (**Chapter 2**). Incubation temperatures ranging from 60°C to 70°C distinguished high-temperature *Daqu* from the other types. The high incubation temperature correlated with the abundance of thermophilic fungi, especially *Thermomyces lanuginosus*; thus, *T. lanuginosus* can be considered as a biomarker that can be used to screen for high-temperature *Daqu* samples. Medium and low-temperature *Daqu* types were difficult to distinguish because *Bacillus* spp., the predominant bacteria in *Daqu*, can grow in a wide range of temperatures (between 30°C and 55°C). Therefore, maximum fermentation temperatures of 40-60°C are suitable for microbial growth in medium- and low-temperature *Daqu* but cannot be used to distinguish between these types. This can also explain the similarity between microbial community structures in the core and surface fractions of *Daqu* (**Chapter 3**), which have similar temperatures; this more than any other parameter including oxygen level determines microbial community structure.

Bacterial communities in *Daqu* samples are more complex than those of fungi since the former are more tolerant to high temperatures and respond to different thermal regimes by altering community structure. However, this is also affected by other parameters such as pH, moisture, relative humidity, and acidity (**Chapter 4**). Analysis of the relative microbial abundance revealed that the representation of fungi and LAB during the initial stages of *Fen-Daqu* fermentation (stages 1, 2, and 3) was better correlated with pH, acidity, and moisture content than with incubation temperature. In addition, factors such as the choice of raw materials and geographical location also played an important role, suggesting that *Daqu* samples containing the same substrates and originating from similar climate zones have comparable microbial profiles. *Staphylococcus gallinarum* and *Staphylococcus saprophyticus* were present in *Daqu* samples from southern China but were absent in northern samples (**Chapter 2**); these species can therefore be considered as biomarkers of southern *Daqu*. The presence of certain microbial species can also be used along with attribute-specific biomarkers to distinguish between *Daqu* samples from the same liquor factory (i.e., sample-specific biomarkers). For example, *Weissella confusa* constitutes over

half of the bacterial population in *Fen-Daqu*, while present in low abundance in other *Daqu* types. Table 7.1 lists biomarkers that can be used to distinguish between *Daqu* types based on 16S and 26S rRNA gene libraries. The diversity of the biomarkers underscores the differences in microbial profiles among *Daqu* types depending on temperature, moisture content, pH, and acidity, and may facilitate the selection of *Daqu* samples that have the specific properties desired by liquor producers. Although these biomarkers have been selected based on statistical analyses, they should be regarded with caution due to the limited sample sizes and should be validated in future studies with a larger sample set and by using alternative approaches such as DGGE. It is also worth noting that different approaches (sampling methods and/or PCR amplicon choice) can produce variations in the microbial composition measured in the same sample (**Chapters 2** and **3**).

Table 7.1 Potential biomarkers for various types of *Daqu*

Biomarkers	Type	Differentiation	Justification
Weissella confusa	Sample-specific	*Daqu* produced in Factory 5* vs. in other factories	More than half of bacterial communities in *Daqu* from Factory 5 consist of *Weissella confusa*
Staphylococcus gallinarum and *Stapylococcus saprophyticus*	Attribute-specific	Northern vs. southern *Daqu*	*Staphylococcus gallinarum* and *Staphylococcus saprophyticus* were present in all *Daqu* samples from southern China but were absent in northern samples
Thermomyces lanuginosus	Attribute-specific	High-vs. medium/low-temperature *Daqu*	*Thermomyces lanuginosus* was abundant in high-temperature *Daqu*, but was absent in medium/low temperature *Daqu*
Absidia idahoensis and *Rhizomucor pusillus*	Sample-specific	*Daqu* produced in Factory 4 vs. in other factories	*Absidia idahoensis* and *Rhizomucor pusillus* were detected only in *Daqu* from Factory 4

**Daqu* sample codes indicating liquor factory

7.5 Comparison of methods used to analyse microbial diversity

Culture-dependent methods used to detect microorganisms in food are time-consuming, costly, and can yield ambiguous results (Riesenfeld et al., 2004; Xu, 2006); however, they can provide important information about microbial communities that can be confirmed by culture-independent methods such as DGGE (**Chapters 3** and **4**). Moreover, the culture-dependent approach is essential for carrying out investigations on the growth, morphological characteristics, functionality, and activity of microbial species. In this study, three different methods, including an rRNA amplicon sequencing-based culture-dependent approach and two culture-independent approaches, DGGE and cloning, were used to analyse microbial diversity in *Fen-Daqu* (**Chapters 2**-**4**). Thirty-nine bacterial species (including one that was uncultured) and 15 fungal species were thus identified. Only *B. licheniformis*, *Bacillus pumilus*, and *P. kudriavzevii* were detected by all three approaches, indicating that although the culture-independent analyses provide comprehensive profiles of microbial communities present in *Daqu* including uncultivable organisms, a combined methodology is required to identify the dominant species and understand the complexity of microbial ecosystems in natural fermentation products. It should be noted that the choice of the methods could influence the accuracy of the results.

The advantages and disadvantages of the various methods are listed in Table 7.2. Culture-dependent methods not only provide quantitative information about the major microorganisms present in food products and ingredients but also allow the establishment of microbial cultures that can be used for further experiments. Although standard culture-dependent analyses can detect only between 1-10% of all environmental microbiota (Muyzer et al., 1998), our study showed that about 50% of microorganisms in *Daqu* can be detected. This indicates that the detection limit of the culture-dependent approach is highly dependent on the niche that is investigated. Faeces and soil are difficult niches since culture methods require other conditions, whereas culture conditions for foods are more similar to those used in this work, and therefore a higher proportion of microorganisms are expected to be cultivable. Generating clone libraries (a culture-independent approach), can provide additional options for detecting microbial species, although the results depend on the number of clones that are selected; typically, around 90 clones are recommended (Green et

al., 2012) but for fungi, the number of clones must exceed 90 in order to obtain accurate results due to the low cloning efficiency of fungal genes. PCR-DGGE analysis is a rapid method for monitoring microbial succession during food production and assessing fermentation quality; it can also help to identify the predominant microorganism populations and their dynamics in response to environmental changes. In addition, gene fragments can be isolated from a gel, and it is simple to implement and has high reliability. DGGE overcomes the challenge of detecting microorganisms that are present in low numbers (10^3 CFU/ml or g) in a complex ecosystem (Cocolin et al., 2013). The present work confirmed the effectiveness of combining culture-dependent and -independent analytical approaches for the characterization of microbial communities in *Daqu*. Six bacterial species (*Bacillus cereus*, *B. licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *B. subtilis*, and *Lb. plantarum*) and two fungal species (*P. kudriavzevii* and *W. anomalus*) were detected by the culture-dependent approach and DGGE; of these, *B. licheniformis*, *Lb. plantarum*, *P. kudriavzevii*, and *W. anomalus* were the predominant species in *Fen-Daqu*. Thus, culture-dependent analyses combined with DGGE are the best choice for investigating microbial diversity and dynamics in complex fermentation systems.

Table 7.2 Characteristics of methods used to analyse microbial diversity

Method	Advantages	Disadvantages
Culture-dependent combined with rRNA amplicon sequencing	• Relatively inexpensive • Quantitative • Living isolates can be obtained	• Labour-intensive • Detects only cultivable microorganisms
Culture-independent clone library	• Semi-quantitative • High sensitivity of detection • Includes uncultivable microorganisms	• Costly and complex • Labour-intensive • Requires a high number of clones for reliability • Low detection level of fungal species
Culture-independent DGGE	• Semi-quantitative • Can reveal populations that are masked due to low abundance (> 10^3 cfu/ml or g) • Highly sensitive • Enables isolation of gene fragments from the gel • Microbial dynamics during the production process can be visualized and dominant populations can be identified	• Costly • Different organisms can appear as a single band • A single microbial strain can produce more than one band due to multiple rRNA genes

7.6 Recommendations and future directions

A comprehensive analysis of microbial community structure in *Daqu* can modernize traditional technologies and help liquor and vinegar factories improve production efficiency and the quality and safety of their products. The following recommendations are made based on the results of this study.

Development of a Fen-Daqu production process with a reduced time

(1) *Fen-Daqu* is produced by a complex, traditional fermentation process consisting of seven steps: *woqu*, *shangmei*, *liangmei*, *chaohuo*, *houhuo*, *dahuo*, and *yangqu*. There were no significant differences in microbial diversity between stages 4 (*chaohuo*) and 7 (*yangqu*) (total duration, 21 days). *B. licheniformis* comprised > 80% of the bacterial population during stages *chaohuo* and *houhuo*, and fungal diversity was unaltered except for the appearance of *L. corymbifera* after stage 4 (*houhuo*); therefore, the fermentation process can be halted after stage 3 (*liangmei*) to investigate the quality of *Daqu* that can be obtained. In the meantime, another *Daqu* (termed *Fuqu*) can be formulated by adding *B. licheniformis* (6 log CFU/g) and *L. corymbifera* (5 log CFU/g) to the substrates (barley and pea) and incubating at 55°C for 3 days; *Daqu* produced only towards stage 3 and can be combined with *Fuqu* for subsequent alcoholic fermentation. *B. licheniformis* strain 128 has relatively high starch-degrading activity and ethanol tolerance, and can therefore be used directly for this purpose, whereas *L. corymbifera* strains must be subjected to further selection based on enzymatic activity and production of aromatic compounds. However, an excess load of these species can suppress the growth of other functional microorganisms. The present analysis found that the total number of microorganisms in *Daqu*, including bacteria and fungi, was in the range of 7-8 log CFU/g. The correct ratio of microorganisms in the inoculum must be determined before applying this method, which can significantly reduce labour and improve production efficiency.

(2) Fresh *Daqu* is traditionally matured for at least 3 months in order to improve the fermentation ability of *Daqu*. However, maturation time is highly dependent on the type of *Daqu* produced and skills of process operators. A maturation time of ≥ 6 months is needed for some types of *Daqu*, which increases cost and labour. Maturation time can be reduced

by increasing inoculum size. Typically, 10-20% *Daqu* was added to alcoholic fermentation processes; 30% or 50% *Daqu* can be tested along with a shorter maturation time such as 2 or 1 month, so as to reduce production time and cost. However, how this would affect the safety and flavour and overall quality of Chinese liquor should be further investigated.

Development of the Daqu production process by introduction of the "back-slopping" technique

Daqu is normally generated by natural solid-state fermentation, and the products normally are unstable and inconsistent. The quality of *Daqu*-derived products therefore differs significantly and flavours are variable. Therefore, a better regulation of the fermentation process is required to improve the technology of liquor and vinegar production. The "so called back-slopping" technique has been applied to the production of a variety of products such as cheese, beverages, and bread (Holzapfel, 2002; Smid et al., 2014a; Viiard et al., 2013). This practice increases the success rate of natural fermentation and simultaneously allows microbial communities to develop an optimal composition (Smid et al., 2014a). Therefore, there is great opportunity for liquor and vinegar factories to apply back slopping technique to fermentation processes. The effects of back slopping on the stability of microbial composition in *Daqu* and quality of *Daqu*-derived products and the relationship between the number of propagation cycles and the stability of microbial composition in *Daqu* can be investigated with the aim of determining the probability of success when applying the back slopping technique to the production of *Daqu*-derived products.

Fortified fermentation using different functional microorganisms

Adding microorganisms as adjunct cultures at different stages of production is an option for improving alcoholic fermentation. For example, *B. licheniformis* and *Sm. fibuligera* are good starch and protein degraders, and can therefore be added at the start of the production process to ensure efficient degradation of starch and proteins in the raw material and their conversion into soluble compounds that can be used by other microorganisms. *W. anomalus* is a good aroma producer in light-flavour *Daqu* owing to its

active ethyl acetate synthesis; thus, adding *W. anomalus* during the alcoholic fermentation stage can potentially enhance the flavour of the end-product. The addition of specific microorganisms as adjuncts is an attractive approach for both industry and consumers since it would not only upgrade the existing technology but also provide a broader product selection.

Oligonucleotide probes for the detection of dominant species during Daqu production and alcoholic fermentation

Whole-cell fluorescence *in situ* hybridisation using 16S rRNA-targeted oligonucleotide probes is a powerful technique for evaluating phylogenetic identity, morphology, population size, and spatial arrangement of microorganisms in environmental samples (Hugenholtz et al., 2002). The present study identified the predominant microorganisms in *Daqu*, which can be used to design probes for target sequences. The quality of *Daqu* and Chinese liquor varies from one batch to the next; monitoring the growth of dominant species should help improve product consistency. A probe containing the sequences of each identified species can be used to detect growth at any stage of fermentation and can thereby optimize *Daqu* production and improve the quality of *Daqu*-derived products.

Next generation sequencing for the detection of microbial diversity and analysis of gene function

Although three different approaches were employed in the present study to analyse microbial diversity in *Daqu*, the results were consistent only for dominant species and therefore the complete microbial community structure was not determined. The culture-independent approaches had limited sensitivity due to incomplete DNA extraction. Next generation sequencing methods have considerable potential for investigating microbial communities (Davey et al., 2011), as they allow high throughput analysis and acquisition of quantitative and qualitative information about any nucleic acids present in the sample at a relatively low cost (Mardis, 2008). Valuable information can be obtained from genome-wide sequencing and higher level functional analyses that can provide insight into

microbial community dynamics and fermentation activity during *Daqu* production and alcoholic fermentation. This knowledge can be used to establish different starter cultures composed of microbial species mixed in the desired proportions, which can be added prior to alcoholic fermentation. Next generation sequencing may also enable the analysis of potentially harmful contaminating microorganisms, which can help liquor industries improve product safety. Overall, this approach can support technological advancement in commercial liquor production via selection and enrichment of specific microorganisms.

Concluding remarks

As a traditional natural fermentation starter, *Daqu* has a significant impact on the quality and flavour of Chinese liquor and vinegar. An understanding of microbial diversity and functional activity, as well as production dynamics and safety of *Daqu*, can benefit commercial production by allowing the implementation of informed changes to improve and/or expand traditional processes and enhance product quality and safety, which can facilitate entry into international markets. The results of this study show the importance of using multiple approaches for the analysis of microbial dynamics relative to environmental changes. However, only limited data on enzyme activity in *Daqu* were obtained; additional studies of the enzymatic profiles of *Daqu* during different fermentation stages are required to assess the relationship between microbial diversity and metabolite composition, which can contribute significantly to the improvement of Chinese liquor production.

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Summary

Fermented products are important dietary components as staples, adjuncts to staples, condiments, and beverages. China has a long history of producing a wide range of traditional fermented products including Chinese liquor, vinegar, rice wine, and soy sauce, which are a major part of the Chinese economy, valued at about 2000 billion RMB annually. *Daqu* is regarded as a semi-product; it has an essential and critical role in the safety and quality of the final products, as it is involved biochemical changes occurring in raw materials (barley and/or pea), which are vital to the development of precursor compounds that contribute to the flavour and safety of fermented products. This study focused on the microbiological properties and functionality of *Daqu* and the fermentation process.

Chapter 2 describes the characteristics, composition, and manufacture of *Daqu*, a saccharifying and fermenting agent that has a significant impact on flavour. Different types of *Daqu* vary in terms of their maximum fermentation temperature (high, medium, and low) and flavour (sauce, strong, light, and miscellaneous, etc.). *Daqu* consists of microbiota, enzymes, and various chemical compounds (starch, other carbohydrates, proteins, fat, minerals, and amino acids). Its fermentation involves changes in these three components. The manufacture of *Daqu* requires a formulation of ingredients in a proper ratio, grinding and mixing (to increase water absorption), shaping, fermentation (through a succession of major groups of microorganisms), and maturation (equilibrating the chemical composition and microbiological composition). Low-temperature *Daqu* fermentation processes follow a strict temperature regime over the various stages: *Woqu*, *Shangmei*, *Liangmei*, *Chaohuo*, *Houhuo*, *Dahuo*, and *Yangqu*. This results in a succession of microorganisms and the formation of precursors for liquor production.

Microbial communities of different types of *Daqu* were analysed by constructing clone libraries of the 16S and 26S rRNA genes (Chapter 3). Only three species were detected in all types of *Daqu* (*Bacillus licheniformis*, *Saccharomycopsis fibuligera*, and one uncultured bacterium). Various genera of lactic acid bacteria (LAB) were identified in *Daqu* samples, including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus,* and

Weissella. In general, *Bacillus* spp. were found in high abundance and LAB were found in low abundance. *Weissella confusa* was abundant in two *Daqu* samples from factory 7 It was used thus as a biomarker to distinguish the *Daqu* from factory 7 from the products of other factories. *Staphylococcus gallinarum* and *Staphylococcus saprophyticus* were found in southern *Daqu* and were absent in northern *Daqu*, and are thus regarded as location biomarkers (northern or southern China). In general, fungi are more sensitive to heat than bacteria. The fungi *Sm. fibuligera* and *Lichtheimia ramosa* were found in low/medium-temperature *Daqu,* whereas *Thermomyces lanuginosus* occurred in high-temperature *Daqu*. The occurrence of opportunistic pathogens such as *Enterobacter cowanii* and *Escherichia hermannii* indicate a high level of contamination in factory 9, warrantying stricter quality control measures throughout the *Daqu* production process.

In Chapter 4, the occurrence, levels, and diversity of *Fen-Daqu* in the core and outer parts as determined by culture-dependent and -independent approaches were described. Total viable numbers of mesophilic and thermophilic bacteria, LAB, and Enterobacteriaceae in the core and outer parts did not significantly differ at 4-8 log CFU/g, whereas the numbers of bacterial spores were significantly higher in the core of *Daqu*. Total counts of yeasts and moulds in core *Daqu* were about 1 log CFU/g lower than those in the outer *Daqu*. *B. licheniformis* was the predominant bacterial species in *Fen-Daqu*, with yeast species *Sm. fibuligera*, *Wickerhamomyces anomalus*, and *Pichia kudriavzevii*. Mycological media (MEA, DG18, and RBCA) were used for detection of members of the fungal community. RBCA was superior for yeast and mould enumeration. Six genera of moulds were identified in *Fen-Daqu* (*Absidia*, *Aspergillus*, *Mucor*, *Rhizopus*, *Rhizomucor*, and *Penicillium*).

In Chapter 5, the dynamics of the microbiota as a function of changes in environmental factors occurring during *Fen-Daqu* fermentation were investigated. Mesophilic bacteria were dominant at the onset of fermentation. As fermentation progressed, fungi became the predominant members of the microbial community. Lactic acid bacteria followed, resulting in an increase in *Daqu* acidity. *Bacillus* spp. and thermophilic fungi became the dominant groups at the end of fermentation, possibly due to their tolerance to low water activity and high temperature. PCR-based approaches indicated

that members of the genus *Bacillus* comprised approximately 70% of total bacterial isolates. 16S rDNA-PCR-DGGE analysis confirmed that *Bacillus* spp. were ubiquitous throughout the process. Fungal species such as the yeasts *W. anomalus*, *Sm. fibuligera*, and *P. kudriavzevii* were present throughout the fermentation process, while the zygomycetous fungus *L. corymbifera* proliferated only during the final stages of fermentation. Canonical correspondence analysis (CCA) was carried out using the information obtained from DGGE bands, together with environmental variables, to characterize the link between microbial communities and environmental conditions.

Chapter 6 describes the contribution of each dominant species on starch degradation, alcohol production, and flavour formation during alcoholic fermentation. *B. licheniformis*, *Pediococcus pentosaceu*s, *Lactobacillus plantarum*, yeasts *P. kudriavzevii, W. anomalus*, *Saccharomyces cerevisiae*, and *Sm. fibuligera* were isolated from *Fen-Daqu* and the production line, and analysed for starch degradation and alcohol tolerance. In general, bacterial species showed higher starch degrading activities than yeast species. More than 90% of *Sm. fibuligera* strains produced amyloglucosidase and α-amylase. Yeasts showed higher ethanol tolerance than bacterial species. Only a few strains were able to grow on 12% alcohol; most of the yeast species were able to tolerate 8% alcohol, while most of the bacterial isolates were only able to tolerate 4% alcohol. High starch-degrading abilities and high ethanol tolerance were used as the criteria to select bacterial and yeast candidate strains for alcoholic fermentation trials. Therefore, *B. licheniformis* 128, *Ped. pentosaceu*s 6, *Lb. plantarum* 1, *P. kudriavzevii* 12*, W. anomalus 29*, *S. cerevisiae* 1, and *Sm. fibuligera* 4 were blended in various ratios and used for lab-scale alcoholic fermentation trails. Metabolite profiles were determined by ^{1}H-NMR. Significant differences in metabolite production during alcoholic fermentation were observed, and species-specific metabolites were identified by Principal component analysis. *S. cerevisiae* was superior to other species with respect to ethanol production. *S. cerevisiae* and *Ped. pentosaceus* were associated with the decrease of pH. *Sm. fibuligera* and *B. licheniformis* exhibit high amylolytic activities, transforming starch and other polysaccharides into fermentable sugars. Both species contribute little to the aroma of Fen-liquor. *W. anomalus* had positive effects on aroma formation, ethanol production, and inhibition of some mycotoxin-producing fungi such as

Aspergillus and *Penicillium*. *Lb. plantarum* and *Ped. pentosaceus* have different effects on lactate conversion. *Lb. plantarum* converts lactate, while *Ped. pentasaceus* does not. Both species exhibit high amylolytic and proteolytic activities and play important roles in the formation of aromatic compounds in *Fen*-liquor.

In summary, this thesis presents the biodiversity and population dynamics of the microbial community in *Daqu*, a starter culture used for the production of Chinese liquor and vinegar, and examined the role of the major *Daqu* microbiota on subsequent sorghum alcoholic fermentation, with the objective of providing insights that can potentially improve and modernize the existing processes of *Daqu* production. The results obtained demonstrate the importance of using multiple approaches for the analysis of microbial dynamics relative to environmental changes in the *Daqu* production process. Based on these findings, five recommendations are made for optimizing *Daqu* production efficiency and improving product quality and safety.

Samenvatting

Gefermenteerde producten zijn een belangrijk onderdeel van ons dieet, als hoofdbestanddeel van maaltijden, als ingrediënt en in de vorm van dranken. China heeft een lange traditie in het produceren van gefermenteerde producten zoals “Chinese liquor” (een alcoholische sterke drank), azijn, rijstwijn, en sojasaus. De productie van deze traditionele producten is belangrijk voor de Chinese economie. Daqu is een traditionele starter voor alcoholische en azijnfermentaties en is belangrijk voor de kwaliteit en veiligheid van gefermenteerde eindproducten. De samenstelling van de starter is mede bepalend voor de biochemische veranderingen die plaatsvinden in de grondstoffen (gerst en/of erwt). Deze studie richt zich op de microbiologische eigenschappen en functionaliteit van Daqu en het alcoholisch fermentatieproces.

Hoofdstuk 2 beschrijft de karakteristieken, samenstelling, en het productieproces van Daqu. De starter wordt bij verschillende temperaturen geproduceerd (hoog, gemiddeld en laag). Deze verschillen leiden tot duidelijke effecten in de eindproducten, vooral wat betreft smaak en aroma. Daqu bestaat uit een complex mengsel van verschillende micro-organismen, plantaardige en microbiële enzymen, en chemische bestanddelen (zetmeel, andere koolwaterstoffen, eiwit, vet, mineralen en aminozuren). Bij de fermentatie vinden veranderingen plaats van deze drie componenten. Het productieproces van Daqu start met een formulering van de ingrediënten in de juiste verhouding, malen en mengen (om de waterabsorptie te verhogen), vormen, fermentatie (door een opeenvolging van verschillende groepen van microorganismen), en rijping (waarbij de chemische en microbiële samenstelling in evenwicht komt). Lage-temperatuur Daqu-fermentatieprocessen volgen een strikt temperatuurregime over de verschillende stadia: *Woqu*, *Shangmei*, *Liangmei*, *Chaohuo*, *Houhuo*, *Dahuo*, en *Yangqu*. Dit resulteert in een successie in abundantie van verschillende soorten micro-organismen en de vorming van metabolieten die belangrijk zijn voor de liquor-productie.

Met behulp van clone libraries van 16S en 26S rRNA genen is de microbiële samenstelling van verschillende typen Daqu geanalyseerd (Hoofdstuk 3). Slechts drie soorten werden in alle drie types Daqu gevonden (*Bacillus licheniformis*,

Saccharomycopsis fibuligera, en een niet-kweekbare bacterie). Verschillende geslachten van melkzuurbacteriën (MZB) werden geïdentificeerd in Daqu samples, zoals *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, en *Weissella*. Vaak werd *Bacillus* spp. in hoge aantallen gevonden en MZB in lagere hoeveelheden. *Weisella confusa* was oververtegenwoordigd in twee Daqu samples van bedrijf 7. Het kan daarom gebruikt worden als biomarker om de Daqu van bedrijf 7 te onderscheiden van producten van andere bedrijven. *Staphylococcus gallinarum* en *Staphylococcus saprophyticus* werden gevonden in zuidelijke Daqu en waren afwezig in noordelijke Daqu, en kunnen dus als locatie-biomarker gezien worden (noord of zuid China). In het algemeen zijn gisten en schimmels (fungi) gevoeliger voor hitte dan bacteriën. De schimmels *Sm. fibuligera* en *Lichtheimia ramosa* werden in laag/medium-temperatuur Daqu gevonden, terwijl *Thermomyces lanuginosus* voorkwam in hoog-temperatuur Daqu. Het voorkomen van *Enterobacteriaceae* zoals *Enterobacter cowanii* en *Escherichia hermannii* geven een hoog besmettingsniveau aan van bedrijf 9, hetgeen wijst op een noodzaak voor striktere kwaliteitsbeheersingsmaatregelen over het gehele Daqu productieproces.

In Hoofdstuk 4, wordt het vóórkomen, de niveaus en de microbiële diversiteit van Fen-Daqu beschreven in zowel het centrum als de perifere delen van het product, bepaald met kweekafhankelijke en –onafhankelijke methoden. Totale aantallen mesofiele en thermofiele bacteriën, MZB, en *Enterobacteriaceae* in het centrum en de buitenste delen verschilden niet significant bij niveaus van 4-8 log KVE/g, terwijl de aantallen bacteriële sporen wel significant hoger waren in het centrum van Daqu. Totale aantallen gisten en schimmels in het centrum van Daqu waren ongeveer 1 log KVE/g lager dan die van de buitenste rand. *B. licheniformis* was de meest voorkomende bacteriële soort in Fen-Daqu. *Sm. fibuligera*, *Wickerhamomyces anomalus*, en *Pichia kudriavzevii* waren de meest abundante gistsoorten. Mycologische media (MEA, DG18, en RBCA) werden gebruikt voor de detectie van diverse gist en schimmelgemeenschappen. RBCA bleek beter geschikt voor gisten- en schimmeltellingen. Er werden zes schimmelgeslachten geïdentificeerd in Daqu (*Absidia*, *Aspergillus*, *Mucor*, *Rhizopus*, *Rhizomucor*, en *Penicillium*).

In Hoofdstuk 5 wordt de microbiële populatie dynamica beschreven als functie van veranderende omgevingsfactoren gedurende de Fen-Daqu fermentatie. Mesofiele bacteriën

bleken dominant bij het begin van de fermentatie. Tijdens het verloop van de fermentatie werden fungi relatief belangrijkere vertegenwoordigers van de microbiële gemeenschap. Vervolgens namen de melkzuurbacteriën toe, hetgeen resulterende in een toename van de Daqu zuurgraad (lagere pH). *Bacillus* spp. en thermofiele fungi werden de dominante groepen aan het eind van de fermentatie, waarschijnlijk door hun tolerantie tegen lage wateractiviteit en hoge temperatuur. PCR-gebaseerde technieken gaven aan dat soorten van het geslacht *Bacillus* ongeveer 70% van de totale bacteriële isolaten uitmaakten. 16S rDNA-PCR-DGGE analyse bevestigde dat *Bacillus* spp. algemeen voorkwamen gedurende het gehele proces. De gistsoorten *W. anomalus*, *Sm. fibuligera*, en *P. kudriavzevii* konden gedurende het gehele fermentatieproces worden aangetoond, terwijl de schimmel *Lichtheimia corymbifera* alleen tijdens de laatste stappen van de fermentatie tot ontwikkeling kwam. Canonical Correspondence Analysis (CCA) werd gebruikt om de uitgebreide informatie van de DGGE banden en de omgevingsvariabelen te analyseren, om zo een koppeling te maken tussen de microbiële gemeenschappen en de omgevingscondities.

Hoofdstuk 6 beschrijft de bijdrage van elk van de dominante soorten op de zetmeelafbraak, alcoholproductie, en smaakontwikkeling gedurende alcoholische fermentatie. *B. licheniformis*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, de gisten *P. kudriavzevii*, *W. anomalus*, *Saccharomyces cerevisiae*, en *Sm. fibuligera* werden geïsoleerd van Fen-Daqu en de productielijn, en geanalyseerd voor zetmeelafbrekend vermogen en alcoholtolerantie. Over het algemeen toonden de bacteriële soorten hogere zetmeelafbraak dan de gisten. Meer dan 90% van de *Sm. fibuligera* stammen produceerden amyloglucosidase en α-amylase. Gisten toonden hogere ethanoltolerantie dan bacteriële soorten. Slechts enkele stammen konden bij 12% alcohol groeien; de meeste giststammen tolereerden 8% alcohol, terwijl de meeste bacteriële isolaten slechts 4% alcohol tolereerden. Hoge zetmeelafbraakmogelijkheden en hoge ethanoltolerantie werden gebruikt als criteria om bacteriën en gisten te selecteren voor fermentatieproeven. Hiervoor werden *B. licheniformis* 128, *Ped. pentosaceus* 6, *Lb. plantarum* 1, *P. kudriavzevii* 12, *W. anomalus* 29, *S. cerevisiae* 1, en *Sm. fibuligera* 4 gemengd in verschillende verhoudingen en gebruikt voor fermentatie-proeven op laboratorium-schaal. Metabolietprofielen werden bepaald met

proton-NMR. Er werden significante verschillen in metabolietproductie gevonden gedurende de alcoholische fermentatie, en soort-specifieke metabolieten werden geïdentificeerd. *S. cerevisiae* had ten opzichte van de andere species een hogere ethanolproductie. *S. cerevisiae* en *Ped. pentosaceus* konden geassocieerd worden met een afname in de pH. *Sm. fibuligera* en *B. licheniformis* toonden een hoge amylolytische activiteit, die zetmeel en andere polysacchariden omzet in fermenteerbare suikers. Beide soorten dragen weinig bij aan het aroma van Fen-liquor. *W. anomalus* had positieve effecten op de aromavorming, ethanolproductie, en remming van enkele mycotoxine-producerende schimmels zoals *Aspergillus* en *Penicillium*. *Lb. plantarum* en *Ped. pentosaceus* hebben verschillende effecten op de melkzuurconversie. *Lb. plantarum* zet melkzuur om, maar *Ped. pentasaceus* doet dat niet. Beide species tonen een hoge amylolytische en proteolytische activiteit en spelen een belangrijke rol in de vorming van aromatische componenten in Fen-liquor.

Samenvattend toont dit proefschrift de biodiversiteit en populatiedynamica van de microbiota van Daqu, een startercultuur gebruikt voor de productie van Chinese liquor en azijn, en beschrijft de rol van de belangrijkste spelers in de Daqu microbiota en de opvolgende sorghum alcoholfermentatie, met als doel inzicht te verschaffen dat mogelijkerwijs de bestaande Daqu-productie kan verbeteren en moderniseren. De behaalde resultaten laten het belang zien van het gebruik van verschillende methoden voor het analyseren van de microbiële dynamica als functie van veranderingen in heersende omgevingsfactoren in het Daqu productieproces. Gebaseerd op deze resultaten konden vijf aanbevelingen worden gedaan om de Daqu productie-efficiëntie te optimaliseren en de productkwaliteit en veiligheid te verbeteren.

概要

发酵食品在中国历史悠久，分布广泛，是人们日常膳食的重要组成部分，包括主食、副食、饮料以及调味品，其每年生产总值约为 2 万亿人民币，已成为中国经济的一个重要组成部分。中国传统发酵食品以其独特的发酵工艺和理论体系而别具一格，普遍采用“曲”作为发酵剂进行生产，典型代表包括白酒、醋、黄酒和酱油等。大曲是曲的一种，是一种传统固态发酵剂，它在终产品的安全和质量上扮演了一个关键角色，因其涉及到原料（大麦和/或豌豆）的生化反应，大曲对终产品风味的前体物质的生成以及发酵食品安全的提高至关重要。本论文旨在揭示大曲中微生物的特性、功能及其生产过程中微生物菌相此消彼长的演变过程。

第二章综述了大曲的分类、历史、功能及其生产工艺。大曲是酿造过程中的糖化剂和发酵剂，含有大量微生物和酶类，其中所含微生物种类和数量比例直接影响终产品的发酵过程和风味物质的形成。大曲根据所产白酒的香型大致可分为清香型、酱香型、浓香型、兼香型等十二大类，按照其最高品温不同又可分为高温、中温、低温三大类，每一类都有其独特的工艺和特点。大曲是一种天然的发酵基质，众多物质包含其中，归纳起来主要有三类：微生物——菌系，生物催化剂——酶系，风味前体物质和原料——物系。虽然大曲种类繁多，但其发酵原理基本一致。现今，大曲的制作过程已经部分实现了工业化，分为五个步骤：原料配比（根据不同类型的大曲和长期积累的经验来制定原料配比）、粉碎和拌料（增加吸水率、获得均匀的纹理）、成型（形成表面光滑、标准尺寸的模块）、发酵（微生物的繁衍生息），以及贮存过程（平衡化学和微生物组分）。低温大曲的发酵分为卧曲、上霉、晾霉、潮火、大火、后火、养曲 7 个阶段，各阶段都有严格的温度要求，这是微生物菌相演变以及风味前体物的形成过程。

第三章通过构建 16S 和 26S rRNA 基因克隆文库，分析了不同类型的大曲微生物的群落构成。结果表明地衣芽孢杆菌、扣囊复膜酵母和一株未培养的细菌普遍存在于大曲中。另外，乳酸菌在大曲的样品中也被检出，包括肠球菌、乳酸杆菌、明串珠菌、片球菌、链球菌和魏斯氏菌属，但其丰度远远小于芽孢杆菌。混淆魏斯氏菌

在工厂 7 的两个大曲样品中丰度值很高，作为一种特征生物标记物可用于区分工厂 7 和其它工厂的产品。鸡沙门菌和腐生葡萄球菌普遍存在于南方大曲中，而在北方大曲尚未被发现，因此可用作地域生物标记物。在一般情况下，真菌比细菌对热更敏感。扣囊复膜酵母和多枝横梗霉在低/中温大曲中存在，而疏棉状嗜热丝孢菌存在于高温大曲中。病原菌的污染如肠杆菌和赫氏埃希菌表明工厂 9 较之其他厂污染较严重，因此在大曲生产过程中要施行严格的卫生控制措施。

第四章以汾酒大曲为研究对象，采用传统培养和分子生物学相结合的方法对大曲的曲心与曲表微生物数量及其多样性进行了鉴定。实验结果表明：适温菌、嗜热菌、肠杆菌科的总菌数在曲心和曲表并没有显著不同，而细菌孢子的数量在大曲的曲心部分均高于曲表。曲心中霉菌和酵母菌总数低于曲表真菌总数约 1 log CFU/g。地衣芽孢杆菌、异常毕赤酵母、扣囊复膜酵母和东方伊萨酵母是汾酒大曲中的优势菌群。另外，实验比较了三种真菌培养基 (MEA、DG18 与 RBCA) 对大曲中真菌群落组成的检测效果。RBCA 培养基对酵母和霉菌计数是最有效的，共检出六种霉菌：犁头霉、曲霉、毛霉、根霉、毛霉和青霉属。

第五章对汾酒大曲发酵过程中微生物及其环境的变化进行了跟踪调查。适温菌在发酵初期占主导地位；随着发酵的进行，真菌是微生物群落的主要力量；紧接着是乳酸菌，导致大曲的酸度不断增加；芽孢杆菌和嗜热菌由于其强大的耐高温和耐低水活性，在发酵后期迅猛生长。 经研究表明，芽孢杆菌属占总细菌数量约 70%。16S rDNA–PCR-DGGE 指纹图谱鉴定结果证实了芽孢杆菌普遍存在于整个发酵过程。除此之外，真菌如异常毕赤酵母、扣囊复膜酵母和东方伊萨酵母也存在于整个发酵过程，而接合菌--伞状毛霉菌的增殖仅存在于发酵过程的最后阶段。本章还利用典范对应分析(CCA) 对 DGGE 指纹图谱获得的微生物丰度信息与环境变量进行拟合，以此揭示微生物群落结构与环境变化之间的相关联系。

第六章研究了汾酒大曲中优势微生物在酒精发酵过程中的功能，包含淀粉降解、酒精生成以及香味前体形成等。利用从汾酒大曲及其生产线中分离获得的地衣芽孢杆菌、戊糖片球菌、异常毕赤酵母、东方伊萨酵母、植物乳杆菌、酿酒酵母和扣囊复膜酵母对淀粉降解和酒精耐受力进行了检测。结果表明，细菌比酵母菌表现出较

高的淀粉降解活性；植物乳杆菌和戊糖片球菌表现出最高的淀粉降解活性。超过90%的扣囊复膜酵母会分泌淀粉葡萄糖苷酶和 α-淀粉酶；酵母菌比细菌更具酒精耐受力；只有少数菌株能在 12%的酒精培养基中生长，多数的酵母菌能够耐受 8%浓度的酒精，而大多数细菌菌株能耐受 4%浓度的酒精。高淀粉的降解能力和高的酒精耐受力是筛选细菌和酵母进行模拟发酵试验的标准。因此，地衣芽孢杆菌 128、戊糖片球菌 6、植物乳杆菌 1、东方伊萨酵母 12、异常毕赤酵母 29、酿酒酵母 1 和扣囊复膜酵母 4 以各种比例混合，用于实验室规模的发酵试验。由氢核磁共振技术测定酒精发酵过程中各发酵组代谢物组的变化，并利用主成分分析对关键代谢物及其特性进行探究。结果表明，酿酒酵母在乙醇生产中优于其他菌种；酿酒酵母和戊糖片球菌与 pH 值下降相关联，而地衣芽孢杆菌和扣囊复膜酵母表现出较高的淀粉酶活性，可把淀粉和其他多糖转化成可发酵糖但对汾酒的香气贡献不大。而异常毕赤酵母在很多方面起到积极作用，包括香气的形成、乙醇生产，以及对一些产毒真菌如曲霉属和青霉属的抑制作用。植物乳杆菌和戊糖片球菌对乳酸转化的影响不同。植物乳杆菌转化乳酸，而戊糖片球菌不转化。这两个菌株表现出较高的淀粉和蛋白降解能力，并在汾酒香气成分的生成中起着重要作用。

综上，本论文研究了大曲生产过程中微生物多样性与微生物群落的种群动态，通过探讨大曲的微生物组成及其在酒精发酵过程中的功能特征，为提高大曲的质量和安全、推动其生产过程的现代化提供了可靠的科学依据。本研究采用多种方法相结合，揭示了微生物菌群与环境之间的密切关系，基于这些发现，对此提出了五个建议以达到提高大曲生产效率和提高产品质量和安全的目的。

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-Xiao-Wei Zheng-

Wageningen, The Netherlands
May 2015.

List of publications

Peer-reviewed paper

Zheng, X.W., Yan, Z.; Nout, M.J.R., Boekhout, T., Han, B.Z., Zwietering, M.H., Smid, E.J., 2015. Characterization of the microbial community in different types of *Daqu* samples as revealed by 16S rRNA and 26S rRNA gene clone libraries. World Jounal of Microbiology and Biotechnology 31, 199-208.

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. International Journal of Food Microbiology 182-183, 57-62.

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 Microbiology 31 (2), 293-300.

Zheng, X.W., Tabrizi, M.R., Nout, M.J.R., Han, B.Z., 2011. *Daqu* - a traditional Chinese liquor fermentation starter. Journal of the Institute of Brewing 117 (1), 82-90.

Yan, Z., **Zheng, X.W.**, Han, J.S., Han, B.Z., Nout, M.J.R., Chen, J.Y., 2013. Monitoring the ecology of *Bacillus* during *Daqu* incubation, a fermentation starter, using culture-dependent and culture-independent methods. Journal of Microbiology and Biotechnology 23 (5), 614-622.

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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. Journal of Agricultural and Food Chemistry 57 (23), 11354-11359.

Ma, K., Cui, Z.N., **Zheng, X.W.**, Han, J.S., Du, X.W., Chen, J.Y., Han, B.Z., 2011. Diversity of culturable fungi in *Fen-Daqu*. China Brewing (in Chinese) 8, 19-21.

Paper submitted

Zheng, X.W., Yan, Z., Nout, M.J.R., Boekhout, T., Smid, E.J., Zwietering, M.H., Han, B.Z., 2015. Characterization of bacteria and yeasts isolated from traditional fermentation starter (*Fen-Daqu*) through a ^{1}H NMR-based metabolomics approach. (submitted to International Journal of Food Microbiology)

Yan, Z., **Zheng, X.W.**, Han, B.Z., Yan, Y.Z., Zhang, X., Chen, J.Y., 2015. ^{1}H NMR-based metabolomics approach for understanding the fermentation behavior of *Bacillus licheniformis*. (submitted to Letters in Applied Microbiology)

Non peer-reviewed scientific paper

Zheng, X.W., Tabrizi, M.R., Wu, X.H., Chen, J.Y., Boekhout, T., Samson, R., Han, B.Z., Nout, M.J.R., 2008. Chemical and microbiological studies on *Daqu*. The KNAW Symposium: Fungi and Health.

Nout, M.J.R., **Zheng, X.W.**, Han, B.Z., Le, V.D., Wolkers-Rooijackers, J., Samson, R., Boekhout, T., 2011. *Daqu*-a fermentation starter for Chinese liquor fermentation. The 100th anniversary symposium of NVVM.

Curriculum vitae

She was born on 30 April 1981 in Qingdao, Shandong Province, China. In 2007, she obtained her Master of Science degree in Food Technology with a major in Dairy Science and Technology from Wageningen University. She conducted her research for her master's degree on the identification of mastitis-causing pathogens by volatile bacterial metabolites, a project funded by Maxfun. In 2008, she was awarded a fellowship the Royal Netherlands Academy of Arts and Sciences (KNAW) for an international joint research project (07CDP015) between China and the Netherlands. The research focused on understanding the microbial ecology and functionality of *Daqu*, a traditional fermentation starter in China. The results of this project are described in this thesis. Her PhD research was also sponsored by the National Natural Science Foundation of China (No. 31071592) and the National Key Project of the Ministry of Science & Technology of China (2007BAK36B02).

Overview of completed training activities

Discipline specific activities

Courses

Bioprocessing and Food Quality, CAU, Beijing, 2008

KNAW Joint Course-Fungi and Fermentation, CAU, Beijing, 2008

Food Fermentation, VLAG, Wageningen, 2008

Food and Biorefinery Enzymology, VLAG, Wageningen, 2009

Molecular Food Microbiology, LIFE, Denmark, 2009

KNAW Joint Advanced Course-Fungi and Fermentation, CAU, Beijing, 2010

Conferences/Meetings

KNAW Symposium "Fungi and Health", 2008

100th anniversary symposium of NVVM, 2011

Conference on Food Safety and Human Health, 2013

Food Biodiversity Workshop for liquor industry, 2014

General courses

Information Literacy including Endnote Introduction, WGS, 2008

PhD Competence Assessment, WGS, 2009

Project and Time Management, WGS, 2011

Career assessment, WGS, 2011

Advanced Guide to Artworks, 2011

Other activities

Preparation of PhD research proposal

Meetings Laboratory of Food Microbiology, 2008-2012

Laboratory of Food Microbiology PhD trip Japan, 2012

The research work was carried out at the Laboratory of Food Microbiology, Department of Agrotechnology and Food Science, Wageningen University, The Netherlands, and the College of Food Science and Nutritional Engineering, China Agricultural University, China.

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