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Review:

Insights into the microbial diversity and community dynamics of Chinese traditional fermented foods from using high-throughput sequencing approaches*

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Abstract: Chinese traditional fermented foods have a very long history dating back thousands of years and have become an indispensable part of Chinese dietary culture. A plethora of research has been conducted to unravel the composition and dynamics of microbial consortia associated with Chinese traditional fermented foods using culture-dependent as well as culture-independent methods, like different high-throughput sequencing (HTS) techniques. These HTS techniques enable us to understand the relationship between a food product and its microbes to a greater extent than ever before. Considering the importance of Chinese traditional fermented products, the objective of this paper is to review the diversity and dynamics of microbiota in Chinese traditional fermented foods revealed by HTS approaches.

Key words: Chinese traditional fermented foods; Microbiota; High-throughput sequencing
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1 Introduction

Chinese traditional fermented foods have a long history dating back thousands of years (Liu *et al*., 2011). Many kinds of fermented foods have appeared and enjoyed great popularity in China since ancient times. A range of raw materials including grains, vegetables, milk, meat, tea, and beans has been used for fermentation, resulting in a myriad of fermented products like Chinese liquor, traditional aged vinegar, Chinese suancai, and soy sauce. Microbial fermentation not only ensures increased shelf life but also confers other beneficial properties on food in terms of texture, flavor, and nutrition. In other words, food fermentation is not only an effective method for food preservation, but also an economical method for food processing.

It is well known that biochemical conversions occurring during the process of fermentation result from the metabolic activities of microorganisms existing in different ecological niches. Thus, microorganisms, the soul of fermented foods, play a crucial role in determining the overall quality attributes of any fermented food. Therefore, unraveling complex microbial communities of fermented foods and understanding their specific effects on the quality of traditional fermented foods is a topic of continuous attention in food microbiology. Selected strains isolated from traditional fermented foods can be used to standardize their production while maintaining their

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traditional traits. Extensive research has been conducted to uncover the microbial composition of all kinds of Chinese traditional fermented foods and a large variety of microorganisms including archaea, bacteria, and fungi has been revealed. In the past, culture-based methods were the only way to conduct microbiological analysis. These methods are extremely useful in determining the microbial community structure in environments influenced by natural as well as anthropogenic effects. However, they have certain limitations. For instance, many bacterial species lose their ability to grow and switch into a distinct life phase known as the "viable but non-culturable (VBNC)" state, in which they are viable but no longer detectable on the routine bacteriological media on which they would normally grow (Oliver, 2005). In the VBNC state, the metabolic activities and growth rates of bacteria slow down and this is why many of them cannot be detected using culturing methods (Fakruddin *et al.*, 2013). Bacteria stochastically change into the VBNC state as a result of many environmental cues like starvation, stressful pH, temperature shock, oxygen imbalance, and non-optimal salinity (Du *et al*., 2007; Ayrapetyan *et al*., 2015).

Also, some bacteria present in normal and metabolically active form in food or environmental samples do not grow on nutrient-rich agar plates under laboratory conditions. It has been said that microorganisms recovered from the environment by traditional culturing methods are rarely abundant in terms of their number or function in the environment from which they were recovered. Less than 1% of microorganisms are readily culturable and these are considered the weeds of the microbial world (Hugenholtz, 2002). The first evidence of the non-culturable ability of bacteria came from the observation that the number of bacterial cells observed under the microscope did not match the number of colonies that formed on agar plates (Winterberg, 1898). This plate count anomaly was confirmed by Amann (1911). It is estimated that only 30 phyla out of the 100 predicted from phylogenetic analysis contain culturable representative microbes (Alain and Querellou, 2009).

In this context it is important to mention that the terms "uncultured", "uncultivable", and "unculturable" have been used in the literature to describe microorganisms that are not readily cultured under laboratory conditions. These terms are misnomers because upon successful cultivation using novel techniques, these previously unculturable organisms become culturable (Lewis *et al*., 2010). One of the strategies for incubating "uncultured" organisms is to use a diffusion chamber. This tricks bacteria into perceiving that they are growing in their natural environment (Kaeberlein *et al*., 2002). So, the use of the word "unculturable" signifies the fact that our current knowledge of their biology and fastidious growth requirements (pH, nutrients, temperature, osmotic conditions, and many more) is limited. However, efforts toward tailoring synthetic growth media to the imaginable natural growth environment of these bacteria have resulted in great success, making thousands of previously "unculturable" bacteria culturable (Stewart, 2012). Nevertheless, it remains a challenge to bring these fastidious organisms into the laboratory due to a lack of optimal growth conditions on nutrient-rich laboratory media. This deficiency is probably due to a lack of interest and desire in designing and optimizing new growth media, and the availability of fewer trained laboratory personnel with expertise in microbial physiology and growth conditions (Prakash *et al*., 2013).

Based on the above discussion, we conclude that culture-dependent approaches can underestimate the complexity of microbial ecosystems because of bias in selecting the microbes. In contrast, culture-independent approaches, mostly targeting total DNA or RNA rather than relying on traditional cultivation, can provide us with an innovative and comprehensive insight into various microecosystems, including food fermentation systems. During the last two decades, several approaches based on the direct analysis of ribosomal RNA genes have been developed to study directly the genome of environmental microorganisms. These approaches include mainly denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) (Muyzer, 1999), single strand conformation polymorphism (SSCP) (Hayashi, 1991), and terminal restriction fragment length polymorphism (T-RFLP) (Marsh, 1999). Most recently, high-throughput sequencing (HTS) or next-generation sequencing (NGS) approaches (e.g. pyrosequencing) have drawn much attention because of their power for analyzing whole environmental microorganisms, and have been successfully applied to the elucidation of the microbiota of various fermented foods. This approach enables us to understand the

relationship between a food product and its microbes to a greater extent than ever before.

In the field of food fermentation, HTS has been widely employed in profiling microbial ecosystems throughout the world (Bokulich and Mills, 2012; Kergourlay *et al*., 2015). Considering the extensive and promising applications of HTS, this review will focus on the use of HTS methods and their roles in unraveling the complex microbial ecosystems of Chinese traditional fermented foods.

2 High-throughput sequencing and its application to fermented foods

HTS or NGS technology is a revolutionary innovation in the field of gene sequencing and has substantially widened the scope of microbial analysis of environmentally derived samples (Mardis, 2008). It is characterized by a high-throughput data yield from a single run. HTS or NGS is the catch-all term used to describe a number of modern sequencing techniques including Roche/454 technology, Illumina (Solexa) technology, ABI SOLiD technology, Ion torrent: Proton/PGM sequencing and PacBio SMRT (Mayo *et al*., 2014). It has provided new platforms to investigate all kinds of microbial communities and to discover some rare and even unknown species. HTS can generate thousands or even millions of sequences at the same time. These sequences help in identifying rarely culturable or unculturable microbes in a sample. HTS also gives an accurate inventory of all microbial operons and genes that are present or are expressed under certain study conditions (Mayo *et al*., 2014). Compared to DGGE, HTS can provide more detailed information with digitalized results (Ling *et al*., 2010). In the past few years, its application has promptly expanded and recent advances in HTS are revolutionizing the study of complex food microbial communities. A great deal of attention has been given to employing these techniques in a variety of food fermentation ecosystems to investigate the composition, structure, and dynamics of microbial populations, and to discover novel, low-abundant, and taxonomic lineages. Based on this knowledge, we can obtain a comprehensive understanding of the relationship between complex microbial communities and the quality of resulting products.

2.1 Profiling the microbial diversity of fermented foods

The advent of HTS has revolutionized the era of biotechnology by transforming scientific enterprise and allowing the possibility of studying minor details of DNA/RNA that have yet remained beyond our imagination. As a result of recent advances in molecular biology, microbial genomics has changed tremendously and is constantly expanding and deciphering more insights into the uncharted microbial life of tiny creatures. It has been estimated that less than 1% of microbes have yet been cultured. Have we really conquered the microbial world or is it just an illusion? The discovery of metagenomics has made this question obsolete. The first metagenomic studies on microbiota of environmental samples pointed out that 80% of the bacteria identified by metagenomics or by 16S ribosomal RNA (rRNA) pyrosequencing genes had not yet been cultured (Venter *et al*., 2004).

HTS has gained much attention in the field of food microbiology and has provided a myriad of novel technologies to detect and monitor microbes in food, allowing the possibility of determining the molecular mechanisms of food functionality. It is well established that the whole microbial profile cannot be determined using culture-dependent methods (Riesenfeld *et al*., 2004) as they provide a biased picture of overall microbial communities. However, the use of metatranscriptomic approaches, involving the extraction of DNA/RNA directly from a fermented food matrix, has overcome this limitation. In short, DNA or complementary DNA (cDNA, from reverse transcription of RNA) is subjected to polymerase chain reaction (PCR) for the amplification usually of 16S rRNA genes. The use of 16S metagenetic analysis has resulted in a novel description of bacterial consortia associated with cheeses, in which several new bacterial genera, like *Prevotella* and *Arthrobacter*, were reported for the first time (Quigley *et al*., 2012). Metagenomic analyses of various fermented foods like sourdough (Liu *et al*., 2016), kefir (Marsh *et al*., 2013; Nalbantoglu *et al*., 2014), fermented mung beans (Chao *et al*., 2013), Mexican Cotija cheese (Escobar-Zepeda *et al*., 2016), and kimchi (Park *et al*., 2011) have been performed. A recently published review provides detailed information on the application of high-throughput 16S rRNA sequencing to a variety of fermented foods to elucidate microbiota (Kergourlay *et al*., 2015).

2.2 Investigation of the microbial dynamics of fermented foods

Strain monitoring is one of the major applications of HTS in food microbiology. Strain monitoring helps to determine fermentation dynamics, knowledge of which is very important for the proper microbiological risk management of fermented foods. Although most fermented foods are considered safe, some incidents of foodborne infection have been attributed to the consumption of fermented foods (Shaffer *et al*., 1990; Centers for Disease Control and Prevention, 2001). Therefore, monitoring temporal microbial succession, the fate of starter cultures and initial foodborne pathogens during the fermentation process and the final residing microbes of any fermented food is of crucial importance. The food safety of some spontaneously fermented foods can be questioned because of the presence of pathogens in the initial food matrix. HTS has successfully been used to determine the population dynamics of microbial consortia in a variety of fermented foods, including bacterial community temporal succession during the fermentation process of wine grapes (Piao *et al*., 2015), sourdoughs (Weckx *et al*., 2010; Ercolini *et al*., 2013), Mongolian fermented cow's milk (Liu W. *et al*., 2015), mozzarella cheese (Ercolini *et al*., 2012), and kimchi (Jung *et al*., 2011).

2.3 Revealing microbial activities of fermented foods

In the complex microbial diversity of fermented foods, there are a number of technically important microbial strains which impart useful properties to food, like aroma and flavor (Smit *et al*., 2005). It is well known that the performance of a microbe during fermentation can be understood by its gene expression. Metatranscriptomic approaches (the use of messenger and non-coding RNAs) provide important insights into regulatory networks and gene expression during the sampling stage, and in combination with the metaproteome and metabolome, the metatranscriptome provides information concerning the activities and functions of microbial communities (Abram, 2015). Using metatranscriptomic analysis, the global gene expression of microbes during the fermentation process can be determined, which helps to understand the key metabolic activities of microbes and to assess the safety of the process. For instance, the gene expression of lactic acid bacteria (LAB) during the fermentation of kimchi (Jung *et al*., 2013) and sourdough (Weckx *et al*., 2011) was determined using this technique, which helped to determine the functional role of each microbe based on the expression of genes.

3 Application of high-throughput sequencing in Chinese traditional fermented foods

Chinese traditional fermented foods are produced mostly using unique procedures involving complex microbial communities, the profiling of which by traditional culture methods is a great challenge. Food fermentation is a dynamic process involving various endogenous and exogenous factors such as temperature, humidity, and acidity, and as a result, the microbiota involved undergo dynamic changes, which in turn may exert their influence on the fermentation process. Therefore, revealing the dynamics of microbiota is necessary to find out the relationship between microorganisms and the quality and safety of food. Since most of the microbes are difficult to cultivate, traditional culturing remains unsuccessful in determining the microbial community dynamics of any fermented food. However, HTS, based on its powerful ability to decipher complex microbial communities, has gradually been applied by researchers to elucidate the composition and dynamics of microbiota in different kinds of Chinese traditional fermented foods (Table 1) and has already offered unprecedented insights.

3.1 Chinese liquors

In Chinese history, alcohol has been termed the "water of history" because of its role in folklore. The use of alcoholic fermented products has great significance in the dietary culture of China. Among these products, Chinese liquor enjoys unique status in the cultural life of Chinese people. Chinese liquor is one of the six most famous distilled spirits throughout the world. There are many varieties of Chinese liquor based on different production techniques and distinct liquor starters. It is usually produced with the use of a unique starter culture called Daqu, a saccharifying and fermenting agent. Daqu is usually prepared by solid state fermentation from wheat, barley, and/or peas, usually in the form of cakes or bricks. The

Table 1 Studies exploiting HTS to examine the microbial ecology of Chinese traditional fermented foods

Product or fermentation system	Sequencing method or platform	Target gene(s)		Reference
		Bacteria	Fungi	
Daqu for light-flavor liquor	Pyrosequencing	V1–V3 (16S rRNA)		Zhang *et al.*, 2014
Starter of Fen liquor	Pyrosequencing		ITS1	Li *et al.*, 2013
Fermented grains of Fen liquor	Pyrosequencing		ITS1	Li *et al.*, 2011
Cellar mud for strong aromatic liquors	Pyrosequencing	V3–V5 (16S rRNA)		Wang C.D. *et al.*, 2014
Cellar mud for strong aromatic liquors	Illumina MiSeq	V4 (16S rRNA)		Zheng *et al.*, 2015
Sourdough	Illumina HiSeq 2000	V6 (16S rRNA)		Zhang and He, 2013
Sourdough	Pyrosequencing	V3–V5 (16S rRNA)		Liu *et al.*, 2016
Pu-erh tea	Pyrosequencing	Whole genome	Whole genome	Lyu *et al.*, 2013
Pu-erh tea	Pyrosequencing	V1–V3 (16S rRNA)	ITS	Zhao *et al.*, 2015
Zhenjiang aromatic vinegar	Pyrosequencing	V1–V3 (16S rRNA)		Wang *et al.*, 2015
Tianjin Duliu aged vinegar	Illumina	V3 (16S rRNA)		Nie *et al.*, 2013
Tianjin Duliu aged vinegar	Pyrosequencing	V3–V6 (16S rRNA)		Peng *et al.*, 2015
Shanxi aged vinegar	Illumina MiSeq	V4 (16S rRNA)	V4 (18S rRNA)	Li *et al.*, 2015
Shaoxing rice wine	Pyrosequencing	V3–V5 (16S rRNA)		Fang *et al.*, 2015
Shaoxing rice wine	Illumina MiSeq	V4 (16S rRNA)		Liu S.P. *et al.*, 2015
Shaoxing rice wine	Pyrosequencing	V3 (16S rRNA)		Wang P. *et al.*, 2014
Shaoxing rice wine	Illumina HiSeq 2000	Whole genome		Xie *et al.*, 2013
Yoghurt of Xinjiang	Pyrosequencing	V1–V3 (16S rRNA)	V4 (18S rRNA)	Xu *et al.*, 2015
Tarag	Pyrosequencing	V3 (16S rRNA)	ITS1	Sun *et al.*, 2014
Tibetan kefir	Illumina HiSeq 2000	V6 (16S rRNA)		Gao *et al.*, 2013

bricks are subjected to a long period of incubation during which large amounts of enzymes are produced and high microbial loads, including bacteria, yeasts, and molds, develop. Daqu preparation is manipulated under various specific time-temperature control schemes to obtain liquors with distinctive flavors. The most famous flavor types include sauce-flavor (e.g. Maotai liquor), strong-flavor (e.g. Luzhou liquor), light-flavor (e.g. Fen liquor), rice-flavor (e.g. Sanhua liquor), and feng-flavor (e.g. Xifeng liquor). The process generally involves different starters, various fermenting techniques, and distinct geographical environments. Together, these result in different microbial successions during fermentation leading to the production of liquors with distinct flavors. Wang C.D. *et al*. (2014) compared the microbiota of a 10-year-old cellar and a 1-year-old cellar used for the production of Luzhou-flavor liquors using a pyrosequencing approach and concluded that long-term spontaneous batch-fermentation accumulates diverse adaptive microbes belonging to the genera *Aneurinibacillus*, *Bacillus*, *Clostridium*, and *Lactobacillus*, which together produce more flavorful liquors. HTS also showed the prevalence of *Lactobacillus* and *Clostridium* in both 30- and 300-year-old pit muds of Chinese Luzhou-flavor liquors (Zheng *et al*., 2015). On the other hand,

Wickerhamomyces, *Kluyveromyces*, and *Pichia* were the main fungal genera and there was no obvious discrimination within the fungal community in pit muds of different ages. Besides the Luzhou-flavor liquors, the variability of bacteria and fungi present during the fermentation process of Fen liquor was also investigated by HTS. Zhang *et al*. (2014) analyzed the bacterial community of three kinds of Daqu used for Fen liquor brewing using pyrosequencing. Most of the annotated reads were assigned to two phyla, namely Firmicutes and Actinobacteria, and Firmicutes was the most abundant group. LAB were present as an important group in all three samples, accounting for 45.8%, 46.3%, and 24.7% of the bacteria, respectively. Investigation of fungal diversity showed the presence of six families and one genus, i.e. Saccharomycetaceae, Saccharomycopsidaceae, Saccharomycodaceae, Dipodascaceae, Trichocomaceae, Pleosporaceae, and *Candida*, with little variation in composition among different samples (Li *et al*., 2011; 2013). We conclude from all the reports that LAB have a significant influence on the fermentation of different liquors.

3.2 Chinese sourdough

Sourdough is a mixture of flour and water that is fermented by yeast and LAB. It has played a significant role in making flour-based fermented food for a very long time all around the world (Hammes and Gänzle, 1997). It is widely used as a starter for bread making throughout Europe (de Vuyst *et al*., 2002; Ktenioudaki *et al*., 2015) and likewise, is employed as an ideal inoculum in making Chinese steamed bread (Li *et al*., 2014; Zhang *et al*., 2016). Sourdough fermentation significantly improves the quality of the resultant product, in terms of texture, flavor, and nutrition, and prolongs the shelf life by retarding the staling process of starch and decelerating microbial spoilage. Extensive research effort has been directed toward the investigation of the microbiota involved in sourdough fermentation in different European countries using culture-dependent and culture-independent approaches (de Vuyst *et al*., 2014). Generally, both approaches obtained similar outcomes, but culture-independent approaches, especially HTS, often provided more information, like the situation in other fermented foods (Elizaquivel *et al*., 2015). The results showed that yeasts and LAB dominated this specific niche, and *Lactobacillus sanfranciscensis* is regarded as the key sourdough LAB species (Gobbetti and Corsetti, 1997). However, in the case of Chinese traditional sourdough, which has been widely used and distributed around China, there remains a relative lack of knowledge of the microbial composition. Zhang and He (2013) investigated the major bacteria in five sourdough samples collected from different places using the Illumina HiSeq 2000 system and found that *Lactobacillus*, *Leuconostoc*, and *Weissella* were the predominant genera among the five samples. Zhang *et al*. (2011) investigated the diversity of LAB and yeasts in traditional sourdoughs collected from the western region of Inner Mongolia Autonomous Region in China by culturing and PCR-DGGE at the species level. The results showed that *Lactobacillus plantarum* group and *Saccharomyces cerevisiae* were the predominant microflora in their samples. Nevertheless, studies performed by other researchers using pyrosequencing revealed that the dominant LAB species in Chinese sourdough was *L. sanfranciscensis* (Liu *et al*., 2016). This bacterium proved to be the only species present among all fifteen sourdoughs and it accounted for more than half of the bacteria in 9 of the 15 samples. The dominance of *L. sanfranciscensis* in Chinese sourdoughs has also been confirmed by culture-dependent and DGGE approaches (Zhang *et al*., 2015). These conflicting results may be attributed to the different sampling regions and culture conditions used. *L. sanfranciscensis* is fastidious (Kline and Sugihara, 1971), and the conditions applied by Zhang *et al*. (2011) were not appropriate for its recovery. HTS, however, can effectively avoid the bias caused by culturing approaches. Furthermore, pyrosequencing revealed that more than 90% of the bacteria belonged to LAB in 14 of the 15 sourdough samples analyzed. A total of 24 LAB species were found by pyrosequencing, 17 of which were lactobacilli (Liu *et al*., 2016).

3.3 Pu-erh tea

Pu-erh or Pu'er is a fermented dark tea, black to brown in color, with a moderate taste, and is produced only in Yunnan Province, China. The role of Pu-erh tea as an anti-oxidative agent for reducing cholesterol levels and aiding digestion is well acknowledged (Hou *et al*., 2009; Cao *et al*., 2011). There are two main categories of Pu-erh tea, namely raw and ripened

(Tian *et al*., 2013). The processing of raw Pu-erh tea includes the fixation, rolling, and drying of leaves before the tea is compressed into bricks, cakes, or other shapes. The processing of ripened tea involves an extra step called wet piling which is similar to composting and is performed before drying to facilitate the aging process. This step involves fermentation of the leaves in a warm and humid environment by bacterial and fungal cultures. The special flavor and characteristics of this tea result from complex microbial interactions and natural oxidation. Microbiota has a significant influence on the quality of Pu-erh tea, especially during the wet piling. Various kinds of bio-techniques including DGGE (Tian *et al*., 2013; Yang *et al*., 2013), pyrosequencing (Lyu *et al*., 2013), metatranscriptomics (Jiang *et al*., 2012), metagenomics and metaproteomics (Zhao *et al*., 2015) have been used to investigate the microbial composition during the piling process. However, the microbiological profiles of Pu-erh tea are not well understood. There is no doubt that bacteria and fungi cooperate in the piling fermentation, but no consensus on the microbial composition or on which group plays the dominant role in the fermentation process has been reached (Jiang *et al*., 2012; Lyu *et al*., 2013). An integrated metagenomics and metaproteomics investigation of the microbial communities and enzymes in fermentation of Pu-erh tea demonstrated that the dominant bacteria and fungi were Proteobacteria (48.42%) and *Aspergillus* (94.98%), respectively, and that *Aspergillus* was the major host of identified proteins (50.45%) (Zhao *et al*., 2015). Similarly, Jiang *et al*. (2012) compared microbial communities from two different stages during the pile-fermentation of Pu-erh tea using metatranscriptomic analysis and found that *Aspergillus niger* was the overwhelmingly predominant species at both stages. In contrast, a preliminary metagenomic study of Pu-erh tea during pile fermentation by Lyu *et al*. (2013) showed a significantly lower percentage of eukaryota by metagenomic analysis, indicating the dominant role of bacteria in the whole piling fermentation process. Furthermore, yeasts rather than molds accounted for the overwhelming majority of eukaryota. These discrepancies may result from different Pu-erh tea materials and/or different production conditions, or sequencing depths (Lyu *et al*., 2013). In terms of bacterial composition, results vary among different research studies and differences are found when different methods are used. Further research is required to elucidate the microbial successions occurring during the fermentation of Pu-erh tea and to determine the relationship between the microbes and the quality characteristics of the tea.

3.4 Chinese vinegar

Chinese traditional vinegar (CTV) or Cu, also known as cereal vinegar, is not only an important seasoning for Chinese dishes but also a significant element in Chinese daily diet. The production of vinegars in China can date back 3000 years (Shi, 1999). Chinese vinegars are sweeter than western white or cider vinegars. Their average acidity is about 5%–6% with some residues of sugar remaining after fermentation. There is a large variety of Chinese vinegars (including black, white, red, smoky, and herbal) which are categorized based on major or subtle differences in the raw materials, manufacturing process or the nature of the saccharifying agent. Most CTVs are brewed with starchy cereals such as rice, wheat, millet, sorghum, or a combination thereof, by solid-state fermentation that consists mainly of the following stages: preparation of a starter Daqu (if needed, e.g. Shanxi aged vinegar), starch saccharification (SS), alcoholic fermentation (AF), and acetic acid fermentation (AAF). Also, a period of time for aging is often indispensable. Unlike the pure-culture liquid fermentation process of vinegar production in European countries (Tesfaye *et al*., 2002), the fermentation of CTV involves various microbes and complicated but regular successions. Zhenjiang aromatic vinegar, one of the most famous traditional vinegars in China, has a comparatively stable microbial community with a strict series of changes during the fermentation process. Its unique cycle-inoculation style means that the fermentation culture of the 7th day is used as a starter for the next batch inoculation (Xu *et al*., 2011). Results of pyrosequencing showed that during the first three days of the fermentation of Zhenjiang aromatic vinegar, the number of operational taxonomic units (OTUs) dramatically decreased, indicating the elimination of microorganisms intolerant of a stressful environment. The dynamics of *Acetobacter* and *Lactobacillus* in different batches show obvious batch-to-batch uniformity. The relative abundance of *Lactobacillus* shows an initial dramatic

increase followed by a gradual decrease, while the relative abundance of *Acetobacter* keeps rising throughout the whole fermentation process. Thus, there is a trend of succession from *Lactobacillus*-dominated fermentation to *Acetobacter*-dominated fermentation. This succession could be attributable to several environmental stresses: (1) the high level of ethanol in the system supporting the growth of ethanol-resistant acetic acid bacteria; (2) high levels of acetic acid and lactic acid (some species of acetic acid bacteria remain metabolically active in high concentrations of acetic acid); (3) elevated temperature (40–46 °C) selects thermo-tolerant acetic acid bacteria (Wang *et al*., 2015). Another two kinds of typical traditional vinegars in China are Shanxi aged vinegar and Tianjin Duliu aged vinegar, which are distinguished by the use of the starter Daqu, akin to the starter used in Chinese liquor fermentation. Daqu provides multitudinous microorganisms and enzymes for the production of vinegar. In the case of Shanxi aged vinegar, the bacterial and fungal dynamics during the 30-d fermentation processes of Daqu have been investigated by Illumina MiSeq sequencing analysis (Li *et al*., 2015). For bacteria, the results demonstrated that Enterobacteriales and Lactobacillales dominated the whole fermentation process, with their relative abundance dramatically increasing from 7.71% to 62.08% and decreasing from 63.1% to 6.2%, respectively. Streptophyta (Cyanobacteria) accounted for 11.23% initially and then were retrieved at low frequencies (<2% of total sequences) until the end of fermentation. Bacillales were found in low abundance from Days 1 to 19, and then clearly increased at the end of fermentation. All these changes were associated with environmental conditions, such as temperature and moisture. During the fermentation, although the temperature reached 53–60 °C and the moisture content declined to 12%, the relative abundance of Enterobacteriales significantly increased due to their remarkable capacity to adapt to a large span of temperatures and moisture levels (Wei *et al*., 2013). Thermophilic Bacillales can also better survive under harsh conditions with the secretion of various degradative enzymes (Simonen and Palva, 1993). In contrast, Lactobacillales sharply decreased with increasing temperature and remained stable until the end of the fermentation process, and the same was true for Streptophyta (Li *et al*., 2015). The main genera involved in the whole fermentation process include *Erwinia*, *Lactobacillus*, *Weissella*, *Leuconostoc*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Methylotenera*, *Kaistobacter*, and *Acinetobacter*. The fungal communities were less complex than the bacterial communities, with Saccharomycetales, Eurotiales, and Mucorales comprising >99% of all the sequences. Saccharomycetales and Eurotiales dominated the whole fermentation process, while Mucorales accounted for only about 0.1% on the first three days, increasing significantly to 69.22% on Day 7, and dominating until the end of fermentation. Clearly, the fungal communities also change in relation to environmental conditions (Li *et al*., 2015). The genera involved include mainly *Pichia*, *Rasamsonia*, *Amylomyces*, *Saccharomycopsis*, *Saccharomyces*, *Rhizomucor*, *Rhizopus*, *Lichtheimia*, *Wickerhamomyces*, and *Candida*. Taken as a whole, the effects of a high incubation temperature and a low moisture level are selective in inducing a shift of microbial communities during the fermentation of Daqu. Tianjin Duliu aged vinegar is made from sticky rice and red sorghum, which are mixed with Daqu for saccharification followed by the addition of water to allow AF. The whole mixture then goes through AAF. The unique feature of this fermentation (a spontaneous process over 30 d) is the exchange of the upper and lower layers of the mixture in an empty urn on the 16th day. Nie *et al*. (2013) found that four genera namely *Lactobacillus*, *Nostoc*, *Acetobacter*, and *Gluconacetobacter*, whose total abundances were about 95%, showed distinct population dynamics using Illumina sequencing. During the whole AAF process, LAB were much more abundant than other bacteria, with *Lactobacillus* being the major genus. The relative abundances of *Acetobacter*, *Gluconacetobacter*, and *Nostoc* increased as the fermentation progressed. The unique process exerted a special influence on the dynamics of the microbial communities involved. For example, *Acetobacter* increased from 1.30% on the first day to 14.88% on the fifth day. This shows an apparent association between the abundance of this genus and the accumulation of total acids. The population of this genus decreased to 4.28% on the 16th day of fermentation following the exchange of the upper and lower layers. Interestingly, *Nostoc*, *Nocardioides*, *Propionibacterium*, *Pediococcus*, and *Klebsiella* were first found in Chinese traditional

vinegars following the use of HTS, indicating that HTS is more efficient in exploring bacterial communities. Peng *et al*. (2015) analyzed the AAF process by pyrosequencing and the results showed that microbial communities in all samples were dominated by Firmicutes and Proteobacteria. At the genus level, 37 different genera were identified by OTU classification, 9 of which were found to be shared across samples, including *Acetobacter*, *Buttiauxella*, *Lactobacillus*, *Pseudomonas*, *Sphingomonas*, *Swaminathania*, *Bacillus*, *Serratia*, and *Gluconacetobacter*, which may indicate that these were the stable components during AAF.

3.5 Shaoxing rice wine

Shaoxing rice wine, named after the city Shaoxing, has a unique flavor and high nutritional value. It is quite popular among Chinese people and was first recorded more than two thousand years ago. It is an excellent representative of Chinese traditional wines and is internationally well known. Shaoxing rice wine is distinguished by its unique flavor and taste, largely ascribed to its specific microbiota imparted by the starter wheat Qu. Like Daqu used in the fermentation process of Chinese liquor and traditional vinegar, wheat Qu is responsible for the various enzymes and microbes needed during the fermentation process of Shaoxing rice wine. Almost all studies of the microbiota involved in Shaoxing rice wine fermentation have focused on bacteria. Xie *et al*. (2013), employing the Illumina HiSeq 2000 approach, showed a more diverse bacterial composition in a mature wheat Qu than that revealed by the DGGE method. The Qu contained mainly three phyla, namely Actinobacteria, Firmicutes, and Proteobacteria. At the genus level, *Staphylococcus*, *Bacillus*, *Saccharopolyspora*, and *Pantoea* were the most abundant genera, while *Lactobacillus* occurred with a very low relative abundance. Wang P. *et al*. (2014) investigated changes in bacterial diversity during the fermentation of Chinese rice wines using pyrosequencing and the results showed that the bacterial community structures and diversity varied significantly at different fermentation stages. More than ten genera of bacteria were detected in fermentation broth including *Staphylococcus*, *Lactobacillus*, *Pseudomonas*, *Bacillus*, *Saccharopolyspora*, *Weissella*, and a number of uncultured bacteria. *Lactobacillus*, which usually cannot be detected in wheat Qu by DGGE analysis (Guan *et al*., 2012; Zhang *et al*., 2012), but can be found in small amounts by HTS, increases throughout the whole fermentation process eventually becoming the dominant bacterial group (Wang P. *et al*., 2014). *Saccharopolyspora* from wheat Qu has a relatively high abundance with little fluctuation during the process. *Bacillus* also remains relatively stable, albeit at low abundance. Most other genera undergo an initial flourishing stage followed by a continuous diminution, presumably because of their intolerance to the acid environment attributable to LAB. Liu S.P. *et al*. (2015) analyzed bacterial succession during rice wine fermentation using the Illumina MiSeq approach and found that the microorganisms in the fermentation broth belonged to three bacterial phyla, namely Firmicutes, Actinobacteria, and Proteobacteria, with Firmicutes being the dominant phylum. At the genus level, ten dominant genera including *Saccharopolyspora*, *Bacillus*, *Staphylococcus*, *Thermoactinomyces*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Weissella*, *Pseudomonas*, and *Enterobacter* were identified. *Bacillus*, *Staphylococcus*, and *Thermoactinomyces* increased gradually during primary fermentation, and then decreased slightly post fermentation. The first two genera are aerobic and grew well initially due to the high oxygen supply and low alcohol content. However, post fermentation, the depletion of oxygen and increase in alcohol content prevented their growth. *Thermoactinomyces* decreased dramatically due to the low temperature post fermentation. LAB had a different tendency. *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Weissella* decreased sharply at first, then increased and eventually dominated the population by the end of the fermentation. Most of the LAB belonged to facultative anaerobic and acid-tolerant groups. These features helped them to grow better and dominate during the post fermentation stage, which is characterized by low oxygen content and low pH (Liu S.P. *et al*., 2015).

3.6 Fermented dairy products

Fermented dairy products have been consumed in China for thousands of years, especially by nomads. Milk from mammals such as cows, horses, ewes, goats, camels, and yaks can be used to ferment, leading to various fermented dairy products throughout China, e.g. Tibetan kefir, tarag, yoghurt, kumiss, and cheese.

The fermentation process increases the shelf-life of the products, while enhancing the taste and improving the digestibility of milk. These home-made products are fermented spontaneously by natural starter cultures from previous batches having complex microbial compositions (Hu *et al*., 2015; Azat *et al*., 2016). Understanding the microbial diversity and dynamics of these starters is important for the standardization of Chinese traditional fermented milk products. Gao *et al*. (2013) investigated the bacterial diversity in four Tibetan kefir grains from different areas in China using Illumina sequencing. Eleven genera were found: *Lactococcus*, *Lactobacillus*, *Acetobacter*, *Shewanella*, *Leuconostoc*, *Pseudomonas*, *Streptococcus*, *Acinetobacter*, *Pelomonas*, *Dysgonomonas*, and *Weissella*. The microbial composition of Tibetan kefir grains from different areas was found to be similar at the genus level in their study. The bacterial and fungal diversities in tarag, a special naturally fermented dairy product in Mongolia and the northwest of China, were investigated by Sun *et al*. (2014) using pyrosequencing. A total of 47 bacterial and 43 fungal genera in 17 tarag samples were found, with *Lactobacillus* and *Galactomyces* being the predominant genera of bacteria and fungi, respectively. Their results showed that the microbial flora in different samples may be stratified by geographic region. Xu *et al*. (2015) investigated the bacterial and fungal diversities in home-made yoghurts from two counties of Xinjiang Uygur Autonomous Region of China by pyrosequencing. Six bacterial and two fungal phyla, comprising 69 bacterial and 20 fungal genera, respectively, were found among the 22 samples tested. Firmicutes and Ascomycota were the dominant phyla, and as in the tarag samples, *Lactobacillus* was the predominant bacterial genus. However, the predominant fungal genus was *Saccharomyces*, not *Galactomyces* as in tarag. Their results indicated that the microbial communities of home-made yoghurts vary with their geographical origin and manufacturing process.

4 Conclusions and future perspectives

Chinese traditional fermented foods are a significant part of the Chinese dietary lifestyle. However, most traditional foods are still produced empirically and spontaneously due to the lack of knowledge of the microbiota involved in fermentation, which makes it hard to manipulate the fermentation process. HTS has proven to be a powerful tool for exploring complex environmental microbiota and tracking fermentation processes. It has provided a comprehensive view of traditionally fermented foods, laying a foundation for manipulating the process of fermentation by using selected microbes and for improving the overall quality of fermented products while retaining their traditional traits. More traditional fermented foods will be subjected to HTS analysis in the future. However, due to the limitations of reference databases, most sequences may be annotated accurately only at the genus level and many will be aligned to uncultured microbes. Therefore, the reference databases need to be updated to obtain more accurate results, e.g. at the species level. The sequencing of whole genomes rather than 16S rRNA genes, i.e. metagenomics analysis, has also revolutionized the analysis of complex microbial diversities in environmental samples by providing comprehensive information on taxonomic relationships, functional genes, and metabolic pathways. This provides a very useful approach to investigate the relationship between microorganisms and food characteristics. In future, this technique is likely to become sufficiently cheap and accessible for the routine analysis of the microbial diversity of foods.

Compliance with ethics guidelines

Guo-qing HE, Tong-jie LIU, Faizan A. SADIQ, Jing-si GU, and Guo-hua ZHANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：高通量测序在探究中国传统发酵食品菌群多样性及动态变化中的应用

概　要：中国传统发酵食品有着上千年的历史，已经成为中国饮食文化中不可缺少的部分。微生物是发酵食品的灵魂，为了探究传统发酵食品中的微生物组成，多种依赖培养和非培养技术都已应用于不同发酵体系的微生物菌相分析中。高通量测序技术是近几年兴起的生物学技术，它极大地方便了环境微生物多样性的研究，促进了我们对复杂微生物环境的认知，其在发酵食品中的应用提升了我们对发酵食品品质与微生物关系的认识水平。本文综述了高通量测序技术在探究我国传统发酵食品菌相中的应用，总结分析了不同发酵体系中微生物的组成和动态变化。

关键词：传统发酵食品；微生物菌群；高通量测序