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Effect of bioaugmented inoculation on microbiota dynamics during solid-state fermentation of Daqu starter using autochthonous of *Bacillus*, *Pediococcus*, *Wickerhamomyces* and *Saccharomycopsis*

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ABSTRACT

Daqu, a traditional fermentation starter that is used for Chinese liquor and vinegar production, is still manufactured through a traditional spontaneous solid-state fermentation process with no selected microorganisms are intentionally inoculated. The aim of this work was to analyze the microbiota dynamics during the solid-state fermentation process of Daqu using a traditional and bioaugmented inoculation with autochthonous of *Bacillus*, *Pediococcus*, *Saccharomycopsis* and *Wickerhamomyces* at an industrial scale. Highly similar dynamics of physicochemical parameters, enzymatic activities and microbial communities were observed during the traditional and bioaugmented solid-state fermentation processes. Both in the two cases, groups of *Streptophyta*, *Rickettsiales* and *Xanthomonadales* only dominated the first two days, but *Bacillales* and *Eurotiales* became predominant members after 2 and 10 days fermentation, respectively. Phylotypes of *Enterobacteriales*, *Lactobacillales*, *Saccharomycetales* and *Mucorales* dominated the whole fermentation process. No significant difference ($P > 0.05$) in microbial structure was observed between the traditional and bioaugmented fermentation processes. However, slightly higher microbial richness was found during the bioaugmented fermentation process after 10 days fermentation. Our results reinforced the microbiota dynamic stability during the solid-state fermentation process of Daqu, and might aid in controlling the traditional Daqu manufacturing process.

1. Introduction

Fermented foods and beverages are paramount parts of human diet. Various fermented foods are produced based mainly on the addition of starter cultures. Daqu, a mixed culture starter, is an important saccharifying and fermenting agent for the production of traditional Chinese liquor and vinegar (Zheng et al., 2012). Traditional Chinese vinegar, which used as a condiment and preserving agent, health product and medicine in Chinese culture, is brewed through solid-state acetic acid fermentation with the addition of Daqu starter (Li et al., 2015c). Daqu starter contains diverse functional microbes and enzymes, and plays a significant role in the flavor formation of the final vinegar products (Zheng et al., 2011).

Daqu starter is also prepared by an ancient solid-state fermentation technology, which can be dated back to 3000 years ago. Traditionally, Daqu is mostly made from a mixture of barley, wheat and peas, the preparation process of Daqu mainly involves three stages (Zheng et al., 2011): (i) material grinding, mixing and shaping; (ii) spontaneous solid-state fermentation by incubation with controlled temperature; and (iii) drying and ripening. Generally, no selected microorganisms are intentionally added to the fermentation process of Daqu. Up to now, the traditional Daqu starter was produced in an open-work environment with non-autoclaved raw materials, and still inoculated with the matured Daqu from the preceding year, and the composition of microbial communities must undergo and adapt complex physicochemical changes, including moisture, temperature, pH and acidity, during the entire spontaneous solid-state fermentation process of Daqu, these might make the fermentation process neither predictable nor controllable and trigger the final products with inconsistent quality. Alternatively, previous studies have demonstrated that a mixed starter culture is

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often a better choice to predict and control the fermentation (García-Aguirre et al., 2009; Pérez-Chabela et al., 2013).

Nowadays, the use of selected starters is a common practice to accelerate and steer fermentation process, and predict the quality of the final products. A great species have been developed as starters for the fermentation of various foods, such as members of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Staphylococcus* inoculated in naturally sausages and vegetable fermentation (Chang and Chang, 2010; Kargozari et al., 2014; Morot-Bizot et al., 2006), *Bacillus* members of *Bacillus subtilis*, *B. licheniformis* and *B. amyloliquefaciens* selected for the fermentation of traditional Chinese sesame-flavored liquor and African fermented okpehe (Oguntoyinbo et al., 2007; Wu et al., 2014), native strains of *Saccharomyces cerevisiae* and *Acetobacter malorum* inoculated in strawberry vinegar fermentation (Hidalgo et al., 2013), halotolerant microorganisms of *Zygosaccharomyces rouxii*, *Candida versatilis* and *Tetragenococcus halophilus* inoculated in chum salmon sauce mash fermentation (Yoshikawa et al., 2010). Commonly, microbiota dynamics during these fermentations inoculated with selected starters were obviously changed compared to the uninoculated control. For examples, spontaneous kimchi fermentation using naturally *Leuconostoc mesenteroides* resulted in increase of the *Leuconostoc* proportions and decrease of the *Lactobacillus* proportions (Jung et al., 2012), and in the spontaneously acidified sausage fermentation, the species diversity was annihilated when *Staphylococcus carnosus* was added as a starter culture, although not contributed to the aroma profile (Janssens et al., 2012). However, no significant difference in microbiota was also observed among batches inoculated with a mix of *Lactobacillus sakei* strain and a strain of *St. equorum*, *St. epidermidis* or *St. saprophyticus* as starter cultures in a traditional Spanish Galician chorizo fermentation (Fonseca et al., 2013), and batches inoculated with 7-day-old acetic acid fermentation (AAF) vinegar cultures in AAF process of traditional Zhenjiang aromatic vinegar (Wang et al., 2015b).

Recently, members of *Bacillus*, lactic acid bacteria (LAB) including *Lactobacillus*, *Pediococcus* and *Weissella*, and yeasts including *Wickerhamomyces* and *Saccharomycopsis*, were detected as the predominant species and were considered as the functional groups, and could secrete various degradative enzymes and produce flavor compounds in various types of Daqu starters (Li et al., 2014; Nie et al., 2013; Zheng et al., 2014). For examples, *B. subtilis*, *B. licheniformis* and *S. fibuligera* were considered as the strong amylase and β-glucosidase producers (Chi et al., 2009; Zheng et al., 2012), and possessed the ability of degrading starch; *Wickerhamomyces anomalus* was known as ester-producing yeast, which in combination with LAB of *Pediococcus pentosaceus*, has been associated with the production of flavor (Nout, 2009). The aim of this study was to evaluate the effect of bioaugmented autochthonous starter cultures, consisting of a mix of leaching solutions of Daqu powder from the preceding year and autochthonous combinations of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *P. pentosaceus*, *S. fibuligera* and *W. anomalus*, on the microbiota dynamics during traditional solid-state fermentation of Daqu starter, by Illumina-based high-throughput sequencing and quantitative PCR (qPCR) analyses. In addition, the evolution of physicochemical parameters were also determined throughout the whole fermentation process. To our knowledge, this report is the first on the bioaugmentation for traditional fermentation of Daqu starter using the autochthonous *Bacillus*, *Pediococcus*, *Saccharomycopsis* and *Wickerhamomyces*.

2. Materials and methods

2.1. Preparation of inoculum

The traditional inoculum was collected from the leaching solutions of 7000 g Daqu powder from the preceding year after soaked in 50 L for 3–4 h at room temperature. The bioaugmented inoculum was prepared by mixing autochthonous pure cultures and the traditional inoculum as following: three *Bacillus* strain including *Bacillus subtilis* strain I-8 (GenBank number: KJ123709), *Bacillus amyloliquefaciens* strain I-2 (GenBank number: KP119809) and *Bacillus licheniformis* strain I-4 (GenBank number: KP119811), one LAB of *Pediococcus pentosaceus* strain I-12 (GenBank number: KP119819) and two yeasts of *Saccharomycopsis fibuligera* strain I-13 (GenBank number: KP119820) and *Wickerhamomyces anomalus* strain XF-22 (GenBank number: KU923324), previously isolated in our laboratory from traditional Chinese Daqu starters (Li et al., 2014, 2015a), were used for the inoculation as starter cultures. *Bacillus* members were cultured in 200 mL LB broth (1% tryptone (Oxoid), 0.5% yeast extract (Oxoid), 1% NaCl) at 37 °C for 12 h to get appropriate inocula (~10^8 cfu mL^{-1}). *P. pentosaceus* was cultured in 200 mL MRS broth (Difco, USA) at 30 °C for 24 h in an initial population of 6.6 × 10^9 cfu mL^{-1}. *S. fibuligera* and *W. anomalus* starters were grown in 200 mL YPD broth (1% yeast extract (Oxoid), 2% tryptone (Oxoid), 2% D-glucose (Panreac, Spain)) at 28 °C for 48 h to get initial population of ~10^8 cfu mL^{-1}. Then, these 200 mL autochthonous pure cultures were pooled and added in 50 L traditional inoculum to obtain the bioaugmented inoculum. Each *Bacillus* species was added to the mixtures with a concentration of 10^6 cfu mL^{-1}, *Pediococcus* strain was added to the mixtures with an amount of 10^7 cfu mL^{-1}, *Wickerhamomyces* and *Saccharomycopsis* strains were added to the mixtures in a level of 10^6 cfu mL^{-1}. The total levels of bacteria, LAB and *Bacillus* in bioaugmented inoculum were about 1.09, 1.07 and 1.13 Log copies mL^{-1} significantly higher ($P < 0.05$) than that in the traditional inoculum (Fig. 3e–g), respectively.

2.2. Solid-state fermentation of Daqu and sampling

Two batches of full-scale industrial experiments of traditional and bioaugmented solid-state fermentation of Daqu, were carried out in the fermentation room of a traditional vinegar production factory in Shanxi province, China. Batches were named according to the starter culture added: (i) S4 batch, using bioaugmented inoculum as inoculation experiment, (ii) S6 batch, using traditional inoculum as control. Initially, cereal grains of approximately 4200 kg barley and 1800 kg wheat were ground and mixed. After pressed the mixture into brick-shape (28 cm × 18 cm × 5 cm) with the addition of 36%–37% water, Daqu bricks were layer-by-layer stacked in fermentation rooms, and the traditional inoculum and bioaugmented inoculum were layer-by-layer sprayed as inoculation at the surface of Daqu bricks, respectively. After inoculation, the stacked layers of Daqu blocks were incubated and matured for 24 days with strict temperature control, the variation of room temperature and core temperature (pile temperature) of Daqu between the two batches fermentation was controlled similar by forced ventilation according to traditional solid-state fermentation techniques. Daqu samples were collected separately at day 1 (after inoculation) and days 2 (35 °C–42 °C), 5 (45 °C–50 °C), 10 (50 °C–55 °C), 14 (35 °C–45 °C) and 24 (<35 °C) based on the temperature control during the fermentation process (Fig. 1a). To provide adequate representation, Daqu blocks from each stage were randomly selected from the upper, middle, and lower locations in triplicates, which were then ground, mixed, and pooled into sterile Stomacher bags (Stomacher Lab System, London, United Kingdom) to get an experimental Daqu powder sample (approximately 500 g). Also, approximately 200 mL of traditional inoculum and bioaugmented inoculum mixtures were sampled into sterile plastic bottle. All of the samples were immediately stored at −20 °C until further analysis.