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Metagenomics reveals flavour metabolic network of cereal vinegar microbiota

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ABSTRACT

Multispecies microbial community formed through centuries of repeated batch acetic acid fermentation (AAF) is crucial for the flavour quality of traditional vinegar produced from cereals. However, the metabolism to generate and/or formulate the essential flavours by the multispecies microbial community is hardly understood. Here we used metagenomic approach to clarify *in situ* metabolic network of key microbes responsible for flavour synthesis of a typical cereal vinegar, Zhenjiang aromatic vinegar, produced by solid-state fermentation. First, we identified 3 organic acids, 7 amino acids, and 20 volatiles as dominant vinegar metabolites. Second, we revealed taxonomic and functional composition of the microbiota by metagenomic shotgun sequencing. A total of 86 201 predicted protein-coding genes from 35 phyla (951 genera) were involved in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of Metabolism (42.3%), Genetic Information Processing (28.3%), and Environmental Information Processing (10.1%). Furthermore, a metabolic network for substrate breakdown and dominant flavour formation in vinegar microbiota was constructed, and microbial distribution discrepancy in different metabolic pathways was charted. This study helps elucidating different metabolic roles of microbes during flavour formation in vinegar microbiota.

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1. Introduction

Microbial communities are responsible for many existing industrial processes such as multispecies biorefinery (Vanwonterghem et al., 2014) and food fermentation (Bokulich et al., 2014). Traditional food fermentation is one of the oldest and most economical ways of producing and preserving foods which may improve the nutritional value, sensory properties and functional qualities of raw materials (Hugenholtz, 2013). Solid-state acetic acid fermentation (AAF) of traditional vinegar produced from cereals, a key step in producing characteristic vinegar flavours, is a spontaneous mixed-culture process that proceeds in China without spoilage for many centuries (Xu et al., 2011b; Wu et al., 2012). It is also a great model to study the microbial community under selective condition. In an open work environment, microbes that inhabit solid-state vinegar culture (termed *Pei* in Chinese) reproducibly metabolise non-autoclaved raw materials (*e.g.* sorghum, sticky rice, wheat bran) and synthesise flavour compounds (Wang et al., 2015). Thus, the function of reproducible fermentation-based metabolism makes this acidic ecosystem (pH 3.0–3.5) amenable to be adapted for studying the formation and function of microbiota in food fermentation. Recent studies have focused on the diversity and dynamics of the bacterial community in the AAF of cereal vinegars using culture-dependent or culture-independent methods (Nie et al., 2015; Xu et al., 2011a; Wu et al., 2010). Other researchers reported compositions of flavours including organic acids, amino acids, minerals, and volatiles in cereal vinegars (Yu et al., 2012; Chou et al., 2015). However, the mechanisms that underlie the flavour formation by acid-tolerant vinegar microbiota remain poorly characterised. Meanwhile,

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dissecting the metabolic roles of microorganisms in community context remains extremely difficult, wherein the central challenge is the reconstruction of microbial metabolic interaction networks based on environmental genomic information (Hanson et al., 2014).

Here, we adopted the AAF process of Zhenjiang aromatic vinegar, which has been certified with a Protected Geographical Indication (PGI)-European Union (No. 501/2012), as a research model. In the AAF, several nutrients in raw materials including ethanol, starch, glucose (Glc), cellulose, proteins, peptides, amino acids, and inorganic nitrogen can be utilised as the substrates for producing vinegar flavours such as organic acids, amino acids, and volatiles. In this study, dominant flavours in the *Pei* of Zhenjiang aromatic vinegar are determined, and metagenomics is used to evaluate the metabolic potential, distribution, and diversity of microbial members in different biosynthesis pathways of vinegar microbiota.

2. Materials and methods

2.1. Sample collection

Zhenjiang aromatic vinegar, and vinegar *Pei* on the 7th day of AAF were sampled from Jiangsu Hengshun Vinegar Industry Co., Ltd. (Zhenjiang, China). A sterilized cylinder-shaped sampler (Puluody, Xi'an, Shanxi, China) was used to collect *Pei* on the 7th day (about 500 g) from top to bottom at the centre of three parallel AAF pools (0.8 m × 1.5 m × 11 m). Vinegar *Pei* on the 7th day of AAF, containing a mass of functional microorganisms such as *Lactobacillus* and *Acetobacter*, is usually used as the starter to initiate next round fermentation of Zhenjiang aromatic vinegar. Our previous study revealed that the microbial structure of starter among different AAF batches were highly similar (similarity = 90%) (Wang et al., 2015). Thus, we used mixed vinegar *Pei* on day 7 from three parallel AAF pools as a representative sample for metagenomic sequencing. The *Pei* was mixed thoroughly in a sterile plastic bag and immediately stored at −80 °C till further analysis.

2.2. Flavour metabolites analyses

Pei (30 g) was mixed with triple-distilled water (100 mL) in a 250-mL flask by rotational shaking at 100 rpm for 2 h at room temperature and then filtered through a double layer of No. 4 Whatman paper. The extract was used for further analysis.

Contents of nine organic acids (acetic acid, lactic acid, succinic acid, oxalic acid, pyruvic acid, ketoglutaric acid, citric acid, pyroglutamic acid, and tartaric acid) in the vinegar and *Pei* were analysed by HPLC with a Waters Atlantis T3 column (4.6 mm × 250 mm, 5 μm). The vinegar sample (5 mL) or water extract of *Pei* (5 mL) was mixed with 2 mL of zinc sulphate (300 g/L) and 2 mL of potassium ferrocyanide (106 g/L) in a volumetric flask, diluted to 100 mL with distilled water, and then filtrated through a double layer of No. 4 Whatman paper. The filtrate was centrifuged at 10 000*g* for 10 min, and the supernatant was used for organic acid analysis. The mobile phase of HPLC analysis was NaH_2PO_4 (20 mmol/L, pH 7.2), and the column temperature was maintained at 30 °C. UV detection was performed at 210 nm.

Contents of γ-aminobutyric acid and seventeen free α-amino acids (L-alanine (Ala), L-arginine, L-aspartic acid (Asp), L-cystine, L-glutamic acid (Glu), L-glycine, L-histidine, L-isoleucine, L-leucine (Leu), L-lysine, L-methionine, L-phenylalanine (Phe), L-proline (Pro), L-serine, L-threonine, L-tyrosine, valine (Val)) in the vinegar and *Pei* were analysed using HPLC (Agilent 1100, Santa Clara, CA) according to a previous study with modification (Heems et al., 1998). The vinegar sample (5 mL) or water extract of *Pei* (5 mL) was mixed with 5 mL of 10% trichloroacetic acid, and then filtrated through No. 4 Whatman paper. After 10 min of centrifugation (10 000*g*), the supernatant was used for amino acid analysis. A reversed-phase column octadecylsily Hypersil (Agilent, 4.6 mm × 250 mm, 5 μm) was used. Precolumn derivatization of *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate was automatically carried out by HPLC (Agilent 1100, Santa Clara, CA). The column temperature was maintained at 40 °C. The mobile phase A was sodium acetate at 97.5 mmol/L, whereas the mobile phase B was sodium acetate at 48.7 mM/acetonitrile/water at a 1:2:2 ratio (v/v/v). The flow rate was 1.0 mL/min. UV detection was performed at 338 and 262 nm.

Compositions of volatile compounds in vinegar and *Pei* were determined by using headspace solid-phase microextraction/gas chromatography-mass spectrometry (HS-SPME/GC-MS) as previously described (Yu et al., 2012). Mass spectra and retention indices (RI) of compounds detected by GC-MS analysis were compared with published data and those in the MS library of National Institute for Standards and Technology (NIST, Search Version 1.6) and Wiley (NY, 320 k compounds, version 6.0). RI was calculated using a mixture of aliphatic hydrocarbons in accordance with a modified Kovats method. Quantification analysis was done by using 2-octanol as an internal standard.

2.3. Genomic DNA extraction

DNA extraction with the CTAB-based method was used (Zhou et al., 1996). *Pei* (5 g) was mixed with 15 mL of DNA extraction buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB, pH 8.0) and 100 μL of proteinase K (10 mg/mL) in a 50-mL Falcon tube with horizontal shaking at 200 rpm for 30 min at 37 °C. After shaking, 3 mL of 10% SDS was added, and the samples were incubated in a 65 °C water bath for 3 h with gentle end-over-end inversions every 15–20 min. The supernatants were collected after centrifugation at 6000*g* for 10 min at room temperature and transferred into another 50-mL centrifuge tube. The pellets were extracted two more times by adding 4.5 mL of extraction buffer and 1 mL of 10% SDS, vortexing for 10 s, incubating at 65 °C for 10 min, and centrifuging as before. Supernatants from the three cycles of extraction were combined and mixed with an equal volume of chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 vol of isopropanol at room temperature for 1 h. A pellet of crude nucleic acids was obtained by centrifugation at 12 000*g* for 30 min at room temperature, washed with pre-chilled 70% ethanol and resuspended in sterile Tris-EDTA buffer (pH 8.0) to give a final volume of 500 μL. Concentrations of total DNA were measured using a DyNA quant 200 (Hoefer, San Francisco, CA). DNA purity was determined by A260/A280. DNA integrity was verified by 1% agarose gel electrophoresis under ultraviolet light. The DNA was stored at −20 °C till further processing.

2.4. Library construction and sequencing

DNA library preparation followed the manufacturer's instruction (Illumina). We used the same workflow as described elsewhere to perform cluster generation, template hybridization, isothermal amplification, linearization, blocking and denaturisation and hybridization of the sequencing primers. The base-calling pipeline (version IlluminaPipeline-0.3) was used to process the raw fluorescent images and call sequences. We constructed one library (clone insert size 330 bp) for the sample.

2.5. Assembly and gene prediction

High-quality short reads of each DNA sample were assembled by the MetaVelvet (Namiki et al., 2012). In brief, we first filtered the low abundant sequences from the assembly according to 17-mer

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