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 (Vanwonerghem 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. shorgum, 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,
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 (Puludy, Xi’an, Shaanxi, 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 000g for 10 min, and the supernatant was used for amino acid analysis. A reversed-phase column octadecylsilysil Hypersil (Agilent, 4.6 mm × 250 mm, 5 μm) was used. Pre-column 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/acetonitril/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 6000g 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 000g 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 denaturation 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 330bp) 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
frequencies. The 17-mers with depth less than 5 were screened in front of assembly, the low-frequency sequences which were very unlikely to be assembled were removed to significantly reduce memory requirement and make assembly feasible in an ordinary supercomputer.

FragGeneScan, which uses di-codon frequencies estimated by the GC content of a given sequence, and predicts a whole range of open reading frames (ORFs) based on the anonymous genomic sequences, was used to find ORFs from the contigs (Rho et al., 2010).

2.6. Gene functional classification

BLASTx (Altschul et al., 1997) was used to search the gene sequences of the predicted genes against GenBank's non-redundant protein database (NR) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database with e-value < 1 x 10^{-5}. The genes were annotated as the function of the NR or KEGG homologues with lowest e-value. Genes that were annotated by KEGG were assigned into KEGG pathways.

2.7. Taxonomic and functional assignments

Taxonomic and functional assignment of reads was carried out using MetaCV (Liu et al., 2013), which is a composition based algorithm to classify short metagenomic reads (75-100 bp) into specific taxonomic and functional groups. For the functional assignment, reads were mapped with KEGG reference database. As a result, the specific organisms that participate in a pathway could be figured out.

2.8. Bioinformatics analyses and metabolic profile prediction

Enzyme coding genes were mapped into KEGG pathway by KEGG Mapper tools (Kanehisa et al., 2014). A list of coding sequence was submitted to the online service and the matched pathways were coloured on the map. According to the result, a list of metabolic pathways and the enzymes in involved for dominant metabolites including 3 organic acids, 7 amino acids, and 20 volatile compounds in Zhenjiang aromatic vinegar and used for charting flavour biosynthesis networks of vinegar microbiota in the following study.

3.2. Overview of metagenomic data

Totally two separated 9.3 Gbp sequences files were resulted from illumina paired-end sequencer, and 54 340 contigs were generated by MetaVelvet, with a maximum contig length of 132 405 bp and a minimum contig length of 100 bp. The mean contig assembly length was 1015 bp, and the N50 was 1531 bp. The general assembly features of metagenomic sequences are described in Supplementary Table S1. FragGeneScan was used to scan genes located in these assembled contigs, which produced 86 201 predicted protein-coding regions (Supplementary Table S2), with 51 921 (60.2%) being assigned a Genbank NR annotation (with product name) by BLASTx. Among genes annotated with KEGG pathway, 2636 belonged to Metabolism group, 944 belonged to Genetic Information Processing, 538 belonged to Cellular Processes, 527 belonged to Environmental Information Processing, 658 belonged to Human Diseases, and 378 belonged to Organismal Systems. Results are shown in Fig. 2.

3.3. Taxonomic classification of predicted genes

Based on the result of MetaCV, reads were assigned to different phyla and genera as concluded in Fig. 3. Relative abundances of bacteria, euakryote and archaeota in vinegar microbiota were 91.75%, 0.19% and 8.06%, respectively. In the phylum level, Firmicutes (48.16%) and Proteobacteria (6.83%) dominated the microbial community of Zhenjiang aromatic vinegar. In the genus level, a total of 951 genera inhabited in the Pei on day 7. Most of the reads were assigned to Lactobacillus, which took up 44.56% of the total reads (with a number of total reads 6 596 146). The second was Acetobacter, which took up 2.99% of the total reads. The other genus has a relative abundance of less than 1%.

3.4. Predicted metabolic pathways of vinegar microbiota

According to the mapping result of KEGG pathway (Supplementary Fig. S2), predicted metabolic network for the breakdown of 7 substrates and the formation of 20 dominant flavours in the microbial community of Zhenjiang aromatic vinegar was figured out as shown in Fig. 4. Enzymes involved in different metabolic pathways are shown in Table 1. Raw materials for the AAF of Zhenjiang aromatic vinegar consist of wheat bran, rice hull, and rice wine from alcohol fermentation, with a mass ratio of 3.5:1:8.7. Hereinto, ethanol from rice wine is the major substrates for the production of vinegar flavours such as Ace. Besides, hexoses including Glc in the Pei, together with monosaccharides and oligosaccharides that degraded from starch and cellulose in wheat bran and rice hull by microorganisms, were alternative carbon sources for the production of Phe, Tyr, phenylethanol, benzoate, and xylene. The nitrogen source in the Pei, amino acids that were degraded from protein or peptide in raw materials, and inorganic nitrogen including nitrite and nitrate, could be used by microorganisms during the AAF process.
<table>
<thead>
<tr>
<th>MFG</th>
<th>Group name</th>
<th>Pathway in KEGG</th>
<th>EC number of enzymes</th>
<th>Examples (reads &gt; 500)</th>
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<tbody>
<tr>
<td>1</td>
<td>Ethanol utiliser</td>
<td>ko00010</td>
<td>EC1.1.1.1; EC1.1.1.2; EC1.1.2.8</td>
<td>Actinobacteria; Lactobacillus; Acetobacter</td>
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<td>2</td>
<td>Starch degrader</td>
<td>ko000500</td>
<td>EC2.4.1.1; EC2.4.1.2; (T14FZ); EC3.2.1.1</td>
<td>Euryarchaeota; Actinobacteria; Bacteroidetes; Cyanobacteria; Bacillales; Lactobacillus; Leucostocaceae; Clostridia; Rhizobiales; Acetobacter; Rhodocyclales; Alteromonadales; Oseanospirillales; Enterobacteriales; Pasteurellales; δ-Proteobacteria</td>
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<td>Cellulose degrader</td>
<td>ko000500</td>
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<td>Actinobacteria; Clostridia</td>
</tr>
<tr>
<td>4</td>
<td>Glucose utiliser</td>
<td>ko00010; ko00030</td>
<td>EC2.7.1.69; EC2.7.1.122; EC3.2.1.10; EC3.2.1.26; EC4.2.1.126</td>
<td>Actinobacteria; Bacteroidetes; Lactobacillus; Rhodobacteriales; Actinobacteria; Bacillales; Lactobacillus; Leucostocaceae; Streptomycetaceae; Clostridia; Rhodocyclales; Actinobacteria; Elusimicrobia; Rhizobiales; Acetobacter; Enterobacteriales; Pseudomonadales</td>
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<td>5</td>
<td>Hexose (extracellular) utiliser</td>
<td>ko00010</td>
<td>EC1.1.2.3; EC1.1.2.4</td>
<td>Lactobacillales; Bacillales; Actinobacteria; Elusimicrobia; Acetobacter; Burkholderiales; Pseudomonadales</td>
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<tr>
<td>6</td>
<td>Lactate utiliser</td>
<td>ko000620</td>
<td>EC2.7.1.1; EC2.7.1.2</td>
<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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<td>7</td>
<td>Nitrite-Nitrate utiliser</td>
<td>ko000910</td>
<td>EC2.7.1.1; EC2.7.1.2; EC2.7.1.63; EC5.1.3.3; EC6.1.1.47; EC6.1.2.8</td>
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<td>8</td>
<td>Acetate producer</td>
<td>ko000620</td>
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<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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<td>Lactate producer</td>
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<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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<td>10</td>
<td>Phenylalanine-Tyrosine-Pheynlethanol-Benzoate group</td>
<td>ko00360; ko00400</td>
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<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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<td>11</td>
<td>Acetolactoate producer</td>
<td>ko00290</td>
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<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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<td>12</td>
<td>Acetoin-Diacetyl-2,3-Butanediol group</td>
<td>ko00650</td>
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<td>13</td>
<td>3-Methylbutanol-3-Methylbutanic acid-Leucine-Valine group</td>
<td>ko00290</td>
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<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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<td>14</td>
<td>Glutamate producer</td>
<td>ko00250</td>
<td>EC2.7.1.1; EC2.7.1.2; EC2.7.1.63; EC5.1.3.3; EC6.1.1.47; EC6.1.2.8</td>
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<td>15</td>
<td>Proline-Arginine group</td>
<td>ko00330</td>
<td>EC2.7.1.1; EC2.7.1.2; EC2.7.1.63; EC5.1.3.3; EC6.1.1.47; EC6.1.2.8</td>
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<td>16</td>
<td>Alanine producer</td>
<td>ko00473</td>
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<td>Aspartate producer</td>
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<td>18</td>
<td>Succinate producer</td>
<td>ko00020; ko00250</td>
<td>EC2.7.1.1; EC2.7.1.2; EC2.7.1.63; EC5.1.3.3; EC6.1.1.47; EC6.1.2.8</td>
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<td>19</td>
<td>Nonalactone producer</td>
<td>ko01040</td>
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<td>20</td>
<td>Xylose producer</td>
<td>ko00500</td>
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<td>Actinobacteria; Bacteroidetes; Cyanobacteria; Elusimicrobia; Lactobacillus; Rhizobiales; Acetobacter; Burkholderiales; Enterobacteriales</td>
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</table>
Fig. 1. Compositions of organic acids (A), free amino acids (B), and volatile compounds (C) in Zhenjiang aromatic vinegar and Pei.

Fig. 2. Functional diversity of predicted genes that matched the KEGG pathways ($e$-value $\leq 1 \times 10^{-5}$) within microbial community for Zhenjiang aromatic vinegar.
Acetyl-CoA and pyruvate were the central compounds in the metabolic network of vinegar microbiota (Fig. 4). Acetyl-CoA participated the TCA cycle, hexanoic acid formation, and non-alactone biosynthesis. Several organic acids including Suc, 2-oxoglutarate, and 2-oxaloacetate were generated by TCA cycle, which were further transformed to Glu, Asp, Pro, Arg. Pyruvate was an important intermediate for the formation of Lac, Ala, and 2-acetolactate. 2-Acetolactate participated in two metabolic pathways, named as Acetoin-Diacetyl-2,3BDO group and 3MBO-3MBA-Val-Leu group.

3.5. Distribution of microbes in different flavour biosynthesis pathways

Relationship between microorganisms and enzymes in different metabolic pathways is shown in Fig. 5, in which read number of enzyme is correlated with diameter of bubble. The name and read number of enzyme are listed in Supplementary Table S3. Microbial functional groups (MFGs) are defined by their contributions to substrates breakdown and flavour metabolism.

Ethanol might be transformed to acetaldehyde by the co-effect of Actinobacteria, Acetobacter, and Lactobacillus (Table 1). Many genes from archaeota, bacteria, and eukaryote were involved in the degradation process of starch (Fig. 5 and Table 1). Actinobacteria and Clostridia might degrade cellulose in the Pei. Many microorganisms including Actinobacteria, Bacillales, Lactobacillus, Leucoanectaceae, Streptomycesaceae, Clostridia, and Rhodocyclales had potential hexose degradation capacity, and among which, Lactobacillus might be the main user of Glc.

Ace and Lac were the main organic acids in vinegar and Pei. Our results showed that there were two subgroups of potential Ace producer using different substrates. Acetobacter, Actinobacteria,
Bacillales, Burkholderiales, and \(\gamma\)-Proteobacteria might transformed acetaldehyde to Ace by aldehyde dehydrogenase (EC1.2.1.3), while Streptomycetaceae and Lactobacillales related to \textit{Lactobacillus} might produce Ace with pyruvate as the substrate. It is usually considered that \textit{Lactobacillus} is the main Lac producer in cereal vinegar fermentation. In this study, we found many taxons mainly \textit{Acetobacter} might be Lac producer in vinegar microbiota on the 7th day of AAF (Fig. 5), but relative abundance of the enzyme genes in these taxons that involved in the synthetic pathway of Lac were quite low. Our previous study showed that Lac was mainly accumulated in vinegar \textit{Pei} of Zhenjiang aromatic vinegar in the first 9 days of AAF, and its content decreased on the following day as the decrease of \textit{Lactobacillus} in vinegar microbiota (Wang et al., 2015).

Thus, in the \textit{Pei} on the 7th day of AAF, the Lac-forming enzyme genes from \textit{Lactobacillus} were not detected in high relative abundance.

2-Acetolactate has been reported as an important precursor for the biosynthesis of Acetoin-Diacetyl-2,3BDO group and 3MBO-3MBA-Val-Leu group. In vinegar microbiota, many microorganisms participated in the formation of 2-acetolactate (Fig. 5 and Table 1). Actinobacteria, \textit{Lactobacillus}, Mollicutes, \textit{Acetobacter} might participated in the Acetoin-Diacetyl-2,3BDO group, while Euryarchaeota, Actinobacteria, Bacteroidetes, Cyanobacteria, Elusimicrobia, Bacillales, \textit{Lactobacillus}, Clostridia, Rhodobacterales, \textit{Acetobacter}, Burkholderiales, \(\delta\)-Proteobacteria, \(\epsilon\)-Proteobacteria, and Synergistetes might involve in the formation of 3MBO, 3MBA, Val, and Leu.
Amino acids in vinegar Pei might originate from proteins or peptides in raw materials, or synthesised by microorganisms. In Fig. 5 and Table 1, Glu could be synthesised by many microbes in vinegar microbiota, e.g. Actinobacteria and Lactobacillales. Pro and Arg might be mainly synthesised by Actinobacteria, Bacteroidetes, Bacillales, Lactobacillus. Although Ala was a dominant amino acid in Pei, relative abundance of enzyme genes in the biosynthetic pathway of Ala was quite low, indicating Ala might mainly originated from the degradation of proteins or peptides in the substrates.

In the Phe-Tyr-Phenylethanol-Benzoate group, Actinobacteria and Lactobacillus were the main potential phényl ethanol producer by the activity of aryI-alcohol dehydrogenase (EC1.1.1.90), and Phe in the Pei was mainly produced by aspartate transaminase (EC2.6.1.1) from Euryarchaeota, Actinobacteria, Bacteroidetes, Chlorobi, Cyanobacteria, Elusimicrobia, Rhizobiales, Acetobacter, Burkholderiales, and Enterobacteria.

4. Discussion

Previously, many culture-dependent and -independent approaches such as enterobacterial repetitive intergenic consensus (ERIC)-PCR, clone library, and denaturing gradient gel electrophoresis (DGGE) have been used to characterise patterns of microbial community diversity in the AAF of cereal vinegars (Wu et al., 2010, 2012; Xu et al., 2011a; Nie et al., 2015). At the moment, novel sequencing-based tools are pushing forward the culture-independent study of food microbial ecology (Illeghems et al., 2015). In this study, we applied metagenomic shotgun data to unravel a 951 genera-containing picture of the microbial diversity within vinegar Pei, wherein the majority of taxa, such as archaeota related to Crenarchaeota and Euryarchaeota, from vinegar Pei were not reported in previous studies as the inability to isolate by culture-dependent methods. Firmicutes (48.16%) and Proteobacteria (6.83%) dominated the microbial community of cereal vinegar, which is consistent with previous studies (Xu et al., 2011a; Nie et al., 2015). On the other hand, it stands to reason that many shotgun metagenomic sequences in this study could only be annotated in genus-level, and metabolic potentials and ecological roles of any given species in this acidic ecosystem could not be revealed. To this point, we anticipate that the top-down approach of metagenomics, the bottom-up approach of classical microbiology, and single-cell technology will merge to unravel a species-level picture of community assembly in this acidic ecosystem (Huang et al., 2015).

During the AAF process, metabolic role of any given species in the context of community may vary along with fermentation time. According to our previous study, patterns of community assembly in different AAF stages of Zhenjiang aromatic vinegar showed significant difference (Wang et al., 2015). For example, the relative abundance of Lactobacillus dramatically increased on the first day of AAF, and then decreased gradually, while the relative abundance of Acetobacter increased during the whole fermented process. Thus, the bubble size representing the relative abundance of enzyme gene in Fig. 5 will change along with fermentation time. Meanwhile, only the Pei on 7th day of AAF was sampled in this study for metagenomic sequencing. Thus, it is of interest in future study to explore succession of the relationship between taxonomic distribution and enzyme abundance in microbial community of Zhenjiang aromatic vinegar.

In this study, we applied an efficient composition and phylogeny-based algorithm to classify shotgun metagenomic reads from flavour-producing microbiota into specific taxonomic and functional groups, and constructed a metabolic network linking key microbial players driving flavour synthesis. Then, the functional potential of specific taxon involved in the reproducible fermentation-based metabolism of vinegar microbiota could be explored. For example, acetoin, diacetyl, 3MBO, TTMP, 3MBA, and 2,3BDO were dominant volatile compounds in Zhenjiang aromatic vinegar and the Pei (Fig. 1C), while Leu and Val were dominant amino acids (Fig. 1B). Acetoin and diacetyl are precursors for the formation of alkaloid TTMP (termed ligustirazine in China) through the Maillard reaction (Xiao and Lu, 2014), which is a dominant flavour and bioactive compound in Zhenjiang aromatic vinegar (Xu et al., 2011b). Thus, microbes involved in the Acetoin-Diacetyl-2,3BDO group and 3MBO-3MBA-Val-Leu group were quite important for vinegar flavour formation. However, these two MFGs were quite different. Many kinds of microbes participated the 3MBO-3MBA-Val-Leu group, while mainly Actinobacteria, Lactobacillus, Mollicutes, Acetobacter involved in the Acetoin-Diacetyl-2,3BDO group. On the other hand, with the benefit of culture-independent metagenomics approaches, recent studies have successfully defined both taxonomic and the collective gene pool contents of many natural communities (Ishii et al., 2013). However, metagenomics provides no information concerning the dynamic expression and regulation of genes in the environment (Hua et al., 2015). The authors of end-point metagenomes which just tell us which organisms are present in the actual sample and which have been present at some point in the process but may have died and left their DNA are traces in the sample. As such, unravelling the dynamic expression of genes in vinegar microbiota by metatranscriptomic and metaproteomic approaches is ongoing in our lab.

5. Conclusion

By metagenomic analyses, we have figured out the flavour metabolic network in microbiota of Zhenjiang aromatic vinegar, and revealed the distribution discrepancy of microorganisms in different metabolic pathways. Via this approach, besides the microbes responsible for organic acids metabolism, two important microbial functional groups participating in two flavour biosynthesis pathways of Acetoin-Diacetyl-2, 3-Butanediol and 3-Methylbutan-3-Methylbutanoic acid–Leucine–Valine were revealed. Our approach is helpful to elucidate the mechanisms that underlie the metabolites formation in multispecies microbial community of cereal vinegar. Also, metagenomic shotgun sequencing result provide a valuable reference for further genetic studies of vinegar microbiota.

Conflict of interest

The Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.09.010.

References


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