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Analysis of Bacterial Diversity During Acetic Acid Fermentation of Tianjin Duliu Aged Vinegar by 454 Pyrosequencing

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Abstract The vinegar *pei* harbors complex bacterial communities. Prior studies revealing the bacterial diversity involved were mainly conducted by culture-dependent methods and PCR–DGGE. In this study, 454 pyrosequencing was used to investigate the bacterial communities in vinegar *pei* during the acetic acid fermentation (AAF) of Tianjin Duliu aged vinegar (TDAV). The results showed that there were 7 phyla and 24 families existing in the vinegar *pei*, with 2 phyla (*Firmicutes*, *Protebacteria*) and 4 families (*Lactobacillaceae*, *Acetobacteraceae*, *Enterobacteriaceae*, *Chloroplast*) predominating. The genus-level identification revealed that 9 genera were the relatively stable, consistent components in different stages of AAF, including the most abundant genus *Lactobacillus* followed by *Acetobacter* and *Serratia*. Additionally, the bacterial community in the early fermentation stage was more complex than those in the later stages, indicating that the accumulation of organic acids provided an appropriate environment to filter unwanted bacteria and to accelerate the growth of required ones. This study provided basic information of bacterial patterns in vinegar *pei* and relevant changes during AAF of TDAV, and could be used as references in the following study on the implementation of starter culture as well as the improvement of AAF process.

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Introduction

Vinegar, as an important element in dishes and preservative agent, is widely used around the world. Many studies were carried out to investigate the functions of vinegar [22], to provide a new method to monitor the AAF of vinegar [3], and to optimize the fermentation process [24]. Unlike the pure-culture liquid fermentation process for vinegar production in European, traditional solid-state fermentation technology is widely used in China, which includes starch saccharification, alcohol fermentation, and oxidation of ethanol to acetic acid. The fermentation of Chinese vinegar is usually a spontaneous process, with the growth of diverse microorganisms and accumulation of their metabolites. Microbial diversity and its constant changes during the fermentation process are sufficient to affect the characteristics of vinegar. Therefore, investigation of the microbial communities involved in the fermentation is very necessary to understand the microbiological processes and then control vinegar quality. A number of studies analyzing the microorganisms during vinegar fermentation by culture-dependent methods have been published, providing a partial picture of the microbial community [9, 10, 26, 33]. However, culture-dependent approach is deficient for understanding the microflora comprehensively since the obtained results covered only microorganisms that could be easily cultivated [14]. Novel approaches are still needed to provide a better understanding of the complex microbial communities in the process of vinegar production.

Recently, the microbial researches of Chinese traditional vinegar based on culture-independent approaches were subsequently reported, suggesting the existence of a vast undiscovered microbial diversity. Many acetic acid bacterium (AAB) strains from cereal vinegar produced by solid-state fermentation were detected by enterobacterial

repetitive intergenic consensus (ERIC)–PCR fingerprinting [34], PCR-mediated denaturing gradient gel electrophoresis (DGGE) [5, 28], and PCR-temporal temperature gradient gel electrophoresis (TTGE) [13]. PCR–DGGE method was also applied to monitor the microbial communities existing in the solid-state AAF of Chinese vinegar [11, 36]. Although these techniques have detected some previously uncultured microbes, the inventory of the microbes associated with the AAF is thought to be far from complete.

Pyrosequencing, a high-throughput sequencing technique, allows the rapid and accurate sequencing of nucleotide sequences, and then to assess the relative abundance of different microbes in samples obtained from different environments. Currently, this emerging technique has been used to study microbial diversity and metabolic capabilities of microbes in different fermented foods, including soybean pastes, processed meat, kefir grains as well as cheeses [7, 15, 20, 35]. Compared with culture and non-culture methods mentioned above, the result obtained by pyrosequencing could provide more comprehensive microbial information of the samples. For example, Kim et al. [14] applied both pyrosequencing and cultivation-based approaches to explore the bacterial communities in Meju (a Korean traditional fermented soybean brick). The bacterial genera varied from 23 to 561 in different samples using pyrosequencing method, while only 11 genera detected in all samples by cultivation-based approach. Nonetheless, there is no report describing the application of pyrosequencing to reveal the microbial diversity in the fermentation of Chinese vinegar.

TDAV is another popular vinegar in northern China besides Shanxi aged vinegar. The product, rich in various vitamins, amino acids as well as organic acids, is made from sticky rice and red sorghum, and manufactured by the steps as previously reported [21]. Briefly, raw materials are mixed with *Daqu* (a mixed starter culture) for starch saccharification, followed by the alcohol fermentation process after the step of adding water into the mixture. Then the mixture goes on a 30-day AAF process spontaneously. The most remarkable step in AAF is exchanging the upper and bottom half layer of vinegar *pei* in an empty urn at the 16th day. In that study, PCR–DGGE was applied to investigate the composition and succession of microbial communities during AAF of TDAV, recovering 13 genera involved in the process among which lactic acid bacteria (LAB) were the dominant microbes followed by acetic acid bacteria (AAB) [21]. In the present study, we aimed to expose more hidden bacterial groups in AAF of TDAV by 454-pyrosequencing based on 16S rDNA gene, and catalog the associated bacterial diversity in great detail.

Materials and Methods

Sample Collection

The mixture in AAF (called vinegar *pei* in Chinese) was collected from Tianliduliu Aged Vinegar Co., Ltd. (Tianjin, China). In order to monitor the representative compositions and changes of bacterial communities during AAF, three samples of vinegar *pei* from the 3rd, 18th, and 24th day (D3, D18, D24) were obtained from 25 cm site under the leavening surface.

Chemical Analysis of Vinegar *Pei*

A total of 10 g vinegar *pei* were treated with 30 ml sterile ddH_2O and homogenized by shaking in the incubator at 130 rpm for 30 min. Then the samples were submitted to the following chemical determinations: pH measurement using pH-meter (Lei Ci, Shanghai, China); total acidity by titration with 0.1 M sodium hydroxide to the phenolphthalein end point.

DNA Extraction, PCR Amplification, and the Purification of Amplicons

The protocol of DNA extraction consisted of a suspension and lysis step that was performed prior to purification using Stool DNA Kit (Omega Bio-Tek, Norcross, GA). Briefly, approximately 1 g of each sample was homogenized using quartz sand and then re-suspended in extraction buffer (100 mM Tris/HCl, pH 8; 100 mM EDTA; 100 mM Na_3PO_4, 1.5 M NaCl). For the purpose of cell lysis, lysozyme solution was added into the tubes, which were incubated at 37° C for 30 min. Proteolysis was induced by the addition of proteinase K and the incubation was conducted at 37° C for 30 min. Afterwards sodium dodecyl sulpfate (SDS) solution was added and incubated at 65° C for 1 h, followed by a centrifugation step (10, 000 g; 10 min). The supernatant was collected for the purification process. Subsequent steps were followed according to the manufacturer's protocol.

The V3–V6 regions of 16S rDNA were amplified with bar-coded primers on a MyCycler thermal cycler (Bio-Rad Laboratories, California, USA), using 5 U/μl of taq DNA polymerase in a reaction buffer containing 10× Buffer, the mixture of dNTP and sterile ddH_2O. PCR was conducted with the following thermocycler conditions: initial denaturation at 94° C for 4 min; 25 cycles of denaturation at 94° C for 30 s, annealing at 55° C for 30 s, and extension at 72° C for 50 s; and a final extension at 72° C for 10 min. Amplicons were gel purified using an 2% agarose gel and a

SanPrep PCR Purification Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions.

Pyrosequencing and Sequence Diversity Analysis

Amplicon pyrosequencing was performed by a Roche 454 FLX Genome Sequencer plate using Titanium Series according to the manufacturer's instruction. Pyrosequence reads were processed using the QIIME software package, version 1.7.0 [1, 2]. The sequence data were sorted into each sample batch using the 9 bp barcode tag. Sequences were removed from the analysis if they were <200 bp or >1000 bp in length, had a mean quality score below minimum of 25, contained ambiguous bases exceeding limit of 6 bp, had a homopolymer run exceeding 6 bp, or contained mismatches in primer exceeding limit of 2 bp. Quality-filtered sequences were clustered into operational taxonomic units (OTUs) using UCLUST, with a minimum sequence identity threshold of 97 %. If the similarity was below 3 % dissimilarity cut-off value, the sequence read would be assigned to the "unclassified" group. The first sequence within each OTU was chosen as the OTU representative sequence. Taxonomy was assigned to representative sequences from each OTU using Ribosomal Database Project (RDP) classifier with a confidence of 0.8 [4]. Then OTU tables were summarized to generate the relative abundance of taxonomic labels to the phylum, family and genus levels. Additionally, Venn diagrams were generated to compare the bacterial compositions and structure from the genus level.

Microbial diversity was evaluated within samples (alpha diversity) using QIIME. Alpha diversity was measured with the Chao1 algorithm (the estimated number of OTUs in each sample), Shannon index (the evenness of the populations), Phylogenetic diversity (PD, the amount of phylogenetic branch length observed in each sample), and observed species metrics. Rarefaction, to a sub-sampling depth of 3500 sequences per sample, was performed on all samples. Rarefaction curves were generated by plotting the number of species observed on the *y*-axis against the number of sequences sampled on the *x*-axis.

Results

Chemical Characteristics of Vinegar *Pei*

Changes of total acidity and pH value of vinegar *pei* during AAF process are shown in Fig. 1. The content of total acidity increased from 3722.0 to 5735.1 mg/100 g. The pH value decreased slowly from 3.87 to 3.62, negatively correlating with the increase of total acidity.

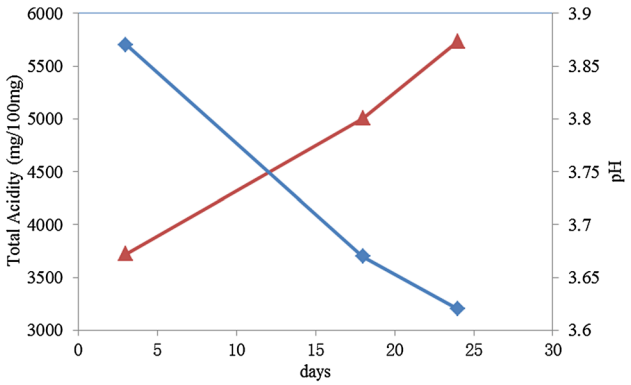

Fig. 1 Analysis of chemical changes in vinegar *pei* during AAF. Total acidity and pH of vinegar *pei* in different stages of AAF (3, 18, and 24 days) were measured and then formed trend lines. *Square*, pH; *triangle*, total acidity

Bar-Coded 454 Pyrosequencing

After quality filtering with QIIME default settings, a total of 21,887 (10,905 for D3, 3987 for D18, and 6978 for D24) sequence reads were recovered through pyrosequencing. The average length of these sequences was approximately 536 bp after trimming the primers. The resulting dataset was subsequently clustered into OTUs by UCLUST (Table 1). Despite each sample containing 323 different OTUs on average, most of these composed a very small portion, while the remaining small number of taxa predominant bacteria there. Averaging across samples, the commonest OTU made up 61 % of all the bacteria sequenced from each sample. Furthermore, the proportion of four most abundant OTUs together exceeded 85 % of all the bacteria. Therefore, the vinegar *pei* has a low bacterial diversity, which was supported by both the number and abundance of OTUs.

Figure 2 shows the rarefaction plots for the three samples, demonstrating that D3 had significantly more phylotypes and greater diversity. Besides, the rarefaction curves of D3 and D18 rise steeply, while that of D24 gently rises and displays a horizontal tendency. As all the rarefaction curves do not level off, it means that the sequencing effort was not large enough to capture the complete diversity of these communities. The Chao1, phylogenetic diversity and Shannon diversity calculated for each dataset are presented in Table 1. Taken together, this suggests that the bacterial communities in D3 were more complex than those in other two samples.

Composition of Bacterial Communities

Different bacterial taxa can have very different effects on the quality of vinegar, so we classified the OTUs to different levels of taxonomy using the RDP pyrosequencing pipeline. Of all qualified reads, 99.4 % could be assigned to

Table 1 Data summary of 454 pyrosequencing analysis, including unique barcode used for different samples, sequencing reads acquired, and comparison of alpha diversity in different vinegar *pei* during AAF

Sample	Barcode[a]	No. of reads analyzed	OTUs[b]	Chao1	Shannon	PD_whole_tree
D3	TATTGACAC	10,905	548	672.267	4.080	8.314
D18	TGACGACAC	3987	268	470.611	4.184	12.597
D24	AGACCTCAC	6978	153	195.960	2.429	5.806

[a] Unique barcode was added into the primer in order to distinguish different samples

[b] The operational taxonomic units (OTUs) were defined with 3 % sequence dissimilarity

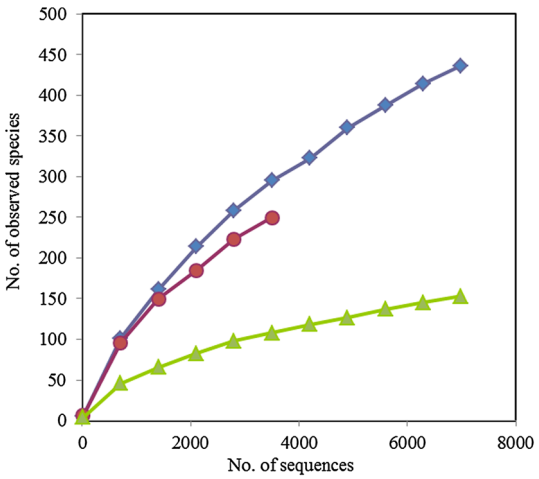

Fig. 2 Rarefaction analysis at the 97 % sequence similarity level. Rarefaction curves were produced by randomly sub-sampling different numbers of sequences from each sample. *Square*, D3; *Circle*, D18; *Triangle*, D24

a known phylum, and 7 different phyla were identified across these samples. Microbial communities in all samples were dominated by *Firmicutes*, which averaged 63.4 % of the communities (ranging from 67.8 to 60.4 % in individual *pei*); and *Protebacteria*, which averaged 29.7 % (ranging from 34.5 to 22.9 %). In the D18, the ratio of *Protebacteria*: *Firmicutes* was close to 1:1.7 whereas in the D3 and D24 the ratio increased (1:2.7 and 1:2.1, respectively), indicating that considerable amount of bacteria belong to *Protebacteria* were involved in vinegar *pei* by the turning-over process at the 16th day. This process also led to a tendency for the relative abundance of *Bacteroidetes* to be greater in D18 (1.5 %) than D3 (0.1 %) and D24 (0.3 %). In contrast, there was a significant decrease in the abundance of *Cyanobacteria* in D18 (2.6 %) compared to D3 (11.8 %), and finally disappeared in D24 due to low tolerance to acids. In general, these four phyla together comprised on average 98.5 % (ranging from 96.7 to 99.8 %) of the reads assigned to the phylum level, forming the core microbiome of vinegar *pei*. In addition, a low prevalence was observed for *Actinobacteria*, *Fusobacteria*, and *Spirochaetes*, which were detected in at least one sample (Fig. 3a).

Family-level analysis focused on the most abundant OTUs, defined as OTUs with a relative abundance >0.1 % in a given sample. Corresponding classification at this level is summarized in Fig. 3b. As shown in Fig. 3b, there were 24 families detected in vinegar *pei* during AAF, and the most abundant families were *Lactobacillaceae* (overall average of 62.4 %), *Acetobacteraceae* (16.6 %), *Chloroplast* (4.8 %), *Enterobacteriaceae* (8.8 %), and *Pseudomonadaceae* (2.7 %). Among these five families, greater abundances of *Enterobacteriaceae* and *Pseudomonadaceae* were observed in D18 than other two samples, whereas the abundance of *Lactobacillaceae* in D18 was found to be the lowest. Similar to that of its corresponding phylum (*Cyanobacteria*), *Chloroplast* prevalence in vinegar *pei* decreased along with the AAF. However, the amount of *Acetobacteraceae* showed an increasing trend along with the fermentation process (ranging from 14 to 19.4 %). A low prevalence was observed for the remaining 19 families (below 1 % in all samples), among which 7 families were detected only in D18. Overall, these results demonstrate a changing microbes in vinegar *pei* during AAF process.

Comparative Analysis of Bacterial Composition Across Samples

Genus-level OTU classification resulted in the identification of 37 different genera, including OTUs with <0.1 % relative abundance. Taken together, OTUs classified to the known 37 genera accounted for over 95 % in any given sample. In order to understand the relatively stable and consistent components during AAF process, the bacterial genera were then analyzed to generate Venn diagrams. Of the 37 different genera identified in vinegar *pei*, 9 genera were found to be shared across samples, 16 genera were found to be unique to D18. Additionally, 8 genera were shared exclusively between D3 and D18, and 4 genera were shared exclusively between D18 and D24 (Fig. 4).

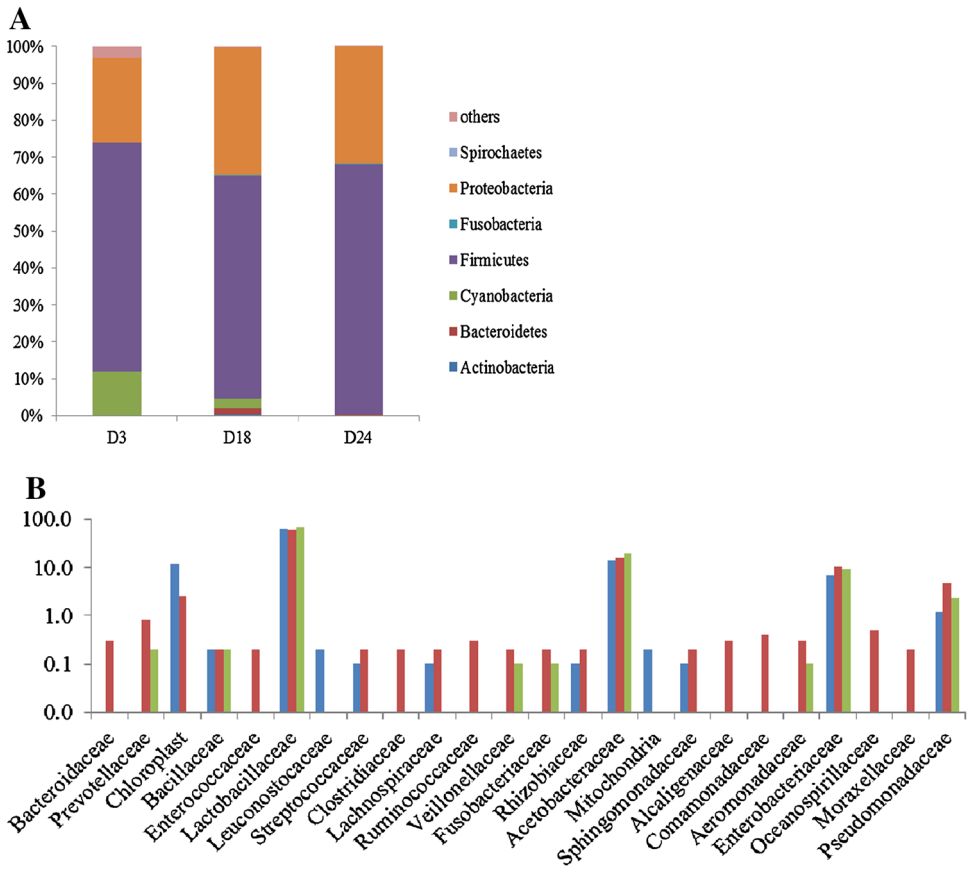

Fig. 3 Analysis of bacterial community composition determined using 454 pyrosequencing. Results of taxonomy at the phylum level and family level were shown in **a** and **b,** respectively. As there were only 4 families with a frequency greater than 10 %, the (**b**) was built by evaluating the corresponding logarithm. *Blue* D3; *Red* D18; *Green* D24 (Color figure online)

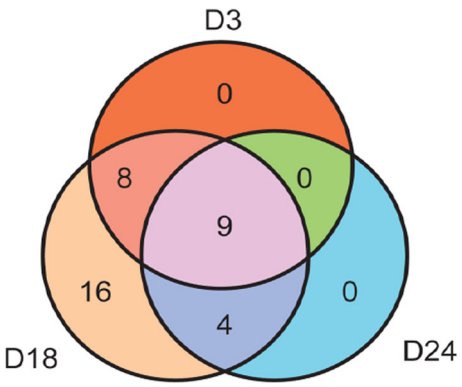

Fig. 4 Comparison of bacterial genera existing in vinegar *pei* during different stages of AAF. Sequences were classified down to lower phylogenetic level. Then Venn diagram was constructed to illustrate the variation of genera in vinegar *pei* during AAF process. These overlapping 9 genera were shared across samples, which indicated that they were the stable components in vinegar *pei* during AAF

With respect to abundance, the vast majority of OTUs were shared across samples, since the relative abundance of 9 genera shared across samples was greater than 80 % (especially amounted to 99 % in D24). In particular, over half of the classified bacteria belonged to *Lactobacillus* (averaging 61.2 %), which was followed by *Acetobacter* (16.6 %) and *Serratia* (7.6 %). Of the 16 genera that were exclusive to D18, there were 6 genera with a relative abundance >0.1 %. They were *Bacteroides* (0.3 %), *Pelomonas* (0.3 %), *Paenalcaligenes* (0.2 %), *Acidaminococcus* (0.2 %), *Oligella* (0.4 %), and *Acinetobacter* (0.2 %). In regard to the 4 genera shared exclusively between D18 and D24 (all below 1 %), there was a tendency toward higher relative abundances in D18 than D24. Since AAF was a successive process, we thought these 20 genera not detected in D3 were brought in the ecosystem through the turning-over process in the 16th day, finally disappearing or decreasing due to the low tolerance to acidic environment. It is worth noting that these 20 genera included genera of known food-borne pathogens, such as *Fusobacterium* reported as the cause of myocardial infection and hematogenous lumbar spondylodiscitis [8, 27], and *Oligella* as a cause of urosepsis and bacteraemia [12, 32]. Therefore, the leaching and sterilizing steps before the packaging of vinegar product are particularly important. Similarly, a low prevalence was observed for the 6 genera shared exclusively between D3 and D18, with *Streptophyta* (4.5 %) and *Pediococcus* (1.4 %) not included. Generally, genera unique to one sample, along with those shared exclusively between two samples, were found to account for a very small minority of the bacteria. The variational abundance of each genus is listed in detail in Table 2.

Discussion

It is critical for the industry of Chinese traditional vinegar to investigate the diversity of bacterial communities involved in the fermentation process, and control them. However, due to the limitation of cultural-dependent method, knowledge regarding the complexity of the microbial communities is still very limited. The aim of this study was to reveal and compare the bacterial community structures in different stages of AAF of TDAV by 454 pyrosequencing.

Pyrosequencing results showed that the two phyla, *Proteobacteria* and *Firmicutes*, were predominant during different AAF stages. *Cyanobacteria* was detected less frequently than the two predominant phyla, presenting a decreasing trend along with fermentation process. Based on the results of previous studies using both culture-dependent and culture-independent methods [5, 11, 13], the three phyla could be considered as typical members involved in the fermentation of traditional vinegar. When the sequences were classified down to lower phylogenetic levels, we found that more genera in vinegar *pei* were revealed by 454 pyrosequencing than other approaches [21, 33, 36], proving that 454 pyrosequencing is more efficient in exploring bacterial communities.

Lactic acid is detected as the second most abundant organic acid in Chinese traditional cereal vinegar after acetic acid [36], which is associated with microbes having the capacity to produce it. In this study, we found several genera of LAB, including *Lactobacillus, Pediococcus*, *Weissella,* and *Streptococcus. Lactobacillus* was the major genera in all the AAF stages, not only lowering the pH enough to inhibit the proliferation of environmental microorganisms, but also enhancing flavor by changing the biochemical conversions. In particular, some strains of *Lactobacillus* were reported for application in foods as natural preservatives due to the production of bacteriocins [16, 17], which could also limit the growth of microbes contaminating the vinegar product. However, a low prevalence was observed for *Pediococcus*, *Weissella,* and *Streptococcus*. Some strains of *Pediococcus* have been reported to have the ability of producing pediocin [29], which could prevent microbial spoilage. *Streptococcus* has been used alone or in combination with *Lactobacillus* for cheese production [19], suggesting its contributions to the mellow flavor of TDAV. To the best of our knowledge, *Streptococcus* was reported for the first time in vinegar *pei*, while structures of *Pediococcus* and *Weissella* were consistent with the previous study [21].

Table 2 Relative abundance at the genus level

Genus	D3	D18	D24	Stage	Genus	D3	D18	D24	Stage
Arcobacter	NA[a]	0.080	0.044	D18&D24	*Pelomonas*	NA	0.295	NA	D18 only
Fusobacterium	NA	0.080	0.044	D18&D24	*Acidaminococcus*	NA	0.200	NA	D18 only
Marinospirillum	NA	0.483	0.029	D18&D24	*Acinetobacter*	NA	0.200	NA	D18 only
Prevotella	NA	0.700	0.058	D18&D24	*Anaerovibrio*	NA	0.100	NA	D18 only
Agrobacterium	0.088	0.215	NA	D3&D18	*Bacteroides*	NA	0.300	NA	D18 only
Aurantimonas	0.039	0.054	NA	D3&D18	*Comamonas*	NA	0.100	NA	D18 only
Enterococcus	0.020	0.215	NA	D3&D18	*Corynebacterium*	NA	0.100	NA	D18 only
Pediococcus	1.398	1.448	NA	D3&D18	*Halomonas*	NA	0.100	NA	D18 only
Roseomonas	0.100	0.200	NA	D3&D18	*Methylobacterium*	NA	0.100	NA	D18 only
Streptococcus	0.068	0.188	NA	D3&D18	*Microbacterium*	NA	0.100	NA	D18 only
Streptophyta	11.734	2.655	NA	D3&D18	*Oceanimonas*	NA	0.100	NA	D18 only
Weissella	0.166	0.027	NA	D3&D18	*Oligella*	NA	0.400	NA	D18 only
Acetobacter	14.000	16.300	19.400	Shared	*Oscillospira*	NA	0.100	NA	D18 only
Buttiauxella	1.183	1.126	1.555	Shared	*Paenalcaligenes*	NA	0.188	NA	D18 only
Lactobacillus	59.400	57.000	67.200	Shared	*Treponema*	NA	0.100	NA	D18 only
Pseudomonas	1.017	4.344	2.195	Shared	*Vibrio*	NA	0.100	NA	D18 only
Sphingomonas	0.039	0.107	0.015	Shared					
Swaminathania	0.068	0.054	0.029	Shared					
Bacillus	0.200	0.100	0.100	Shared					
Serratia	6.200	7.800	8.800	Shared					
Gluconacetobacter	0.300	0.100	0.200	Shared					

[a] Not found in this sample

AAB have the ability to oxidize ethanol to acetic acid, mainly responsible for the elaboration of vinegar. As the results shown, three genera belong to AAB were revealed from the vinegar *pei*, among which *Acetobacter* was the second most abundant genus besides *Lactobacillus*. High ethanol concentration and sufficient oxygen would stimulate the growth of *Acetobacter*. A number of studies have been conducted to identify new strains of *Acetobacter* with high yield [18], to optimize the conditions of fermentation [25], and to develop new mixed culture fermentation for high productivity and low capital investment [30]. Moreover, *Acetobacter pasteurianus* was reported as the most common species in vinegar fermentation manufactured by conventional methods [11, 13, 33, 36], which was also detected as the sole AAB involved in AAF of TDAV [21]. By contrast, we found another two genera of AAB, *Gluconacetobacter* and *Swaminathania*, in low proportions. These two genera were considered as plant growth-promoting bacteria due to their capability of biological nitrogen fixation [23]. Thus, an alternative possibility was that they were nitrogen-resource suppliers of raw material, which then provided energy for the metabolism of functional bacteria.

Compared with results obtained by cultivation-based methods in previous studies, we detected a wider bacterial diversity by 454 pyrosequencing. In terms of LAB, three genera (*Lactobacillus*, *Weissella*, and *Pediococcus*) were isolated from vinegar *pei* [33], while *Streptococcus* was also recovered in this study. AAB isolated from vinegar fermentation were distributed in the genera *Acetobacter* and *Gluconacetobacter* [9], while we also detected *Swaminathania* in addition to those two genera. Thus, as for in-depth analysis and comparisons of bacterial communities in particular environments, the next-generation sequencing technique is able to overcome the limitations of culture study to some degree.

On the whole, the proportion of AAB (averaging 15 %) was much lower than that of LAB (62 %), contrary to the expected results that AAB would be the dominant bacteria because of the ability to produce acetic acid. In European countries, a highly automatic fermentor is commonly used in the submerged pure-culture fermentation of vinegar, which ensures the high efficiency of oxygen uptake during AAF to stimulate the growth of AAB, while providing a stringent environment for the survival of many anaerobic LAB. However, the vinegar *pei* of TDAV exists in solid state and is normally 1 m thick, with AAB growing well in the oxygen-rich upper region and anaerobic LAB breeding in the oxygen-deficient lower region. High abundance of LAB makes great contributions to the accumulation of flavor compounds, leading the difference of tastes between vinegar produced by submerged and traditional techniques. Excess growth of LAB is however undesirable because it causes sugar losses and consequently affects the flavor of the final product. The result could be used as references in the following study on how to balance the corresponding productions and thus improve the quality of vinegar.

Enterobacteriaceae, including the genera *Buttiauxella*, *Erwinia*, *Escherichia*, *Serratia*, was another important group in the vinegar *pei*. For the food fermentation industry, studies of *Enterobacteriaceae* in the food ecosystem of fermented sausages were frequently reported [6], focusing on the strategies for related pathogens control. As this bacterial community was commonly thought to be a positive correlation with the food spoilage, and sometimes involved in food-borne disease outbreaks, it is unexpected to show up in the vinegar *pei* for the ideal. However, due to several factors (e.g., workers, turning-over process, fermentation environment), it makes the appearance of *Enterobacteriaceae* inevitable. In this study, we detected it with a relative abundance of 8.8 %. Nonetheless, there is a step of thermal sterilization before the package of vinegar, resulting in no microbial safety risks for consumers. Despite the possibility that *Enterobacteriaceae* would be a source of contamination in vinegar *pei*, some studies on its beneficial impacts were still reported, e.g., a protease/chitinase-producing strain of *Serratia* was isolated by Wang [31], which could be used as another bacterial source. Therefore, further studies are needed to understand what roles *Enterobacteriaceae* play in the production of vinegar.

The basic requirement to apply 454 pyrosequencing is the availability of a standardized and efficient genomic DNA of the bacteria involved. Based on the culture-dependent result of our previous study (data not shown), we found that there were a great number of *Bacillus*, of which most strains belonged to the species of *B.amyloliquefaciens*, indicating that this bacterial community may make contributions to the process of starch saccharification by secreting amylase. However, the relative abundance of *Bacillus* detected by 454 pyrosequencing was only 0.13 %, much less than we expected. It raises the question of whether the lysing reagents used were efficient enough for the extraction of *Bacillus* DNA. Thus, the protocol of DNA extraction needs to be adapted to recover more bacterial communities.

In summary, this study marked the first attempt to use 454 pyrosequencing to reveal the variations of bacterial communities in different stages of traditional AAF, and the overall microbial diversity of vinegar *pei* was determined. The result enriched the existing knowledge of microbial communities involved in the traditional vinegar fermentation, probably providing basic information to improve the composition of starter culture. Moreover, this study provided novel information for the improvement of process management and for the evaluation of potential

microbiome with impact in other biotechnological applications, and further studies are needed to understand whether these detected genera play a role in improving product quality or act as a possible contamination source.

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