Bacterial diversity in home-made paocai brine of different ages by SMRT sequencing

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Abstract: Paocai is a traditional and popular fermented vegetable food in southwestern China where nearly every household has at least one paocai jar. Paocai brine (fermentation medium of paocai) is often reused for a long time, and some local residents believe the longer age of the brine, the better paocai brine. In order to verify this view, we compared the bacterial diversity in 22 home-made paocai brine (HMPB) samples of different ages (all over 10 years) via a metagenomic approach involving single-molecule, real-time (SMRT) sequencing of the full-length 16S rRNA gene. Our results revealed no significant differences in bacterial diversity and the dominant taxa were observed between HMPB samples within different age groups. But HMPB with longer age may have more metabolic pathways, indicating richer flavor of the HMPB. In general, our results suggested that the not the longer time of HMPB used, the better bacterial profiles of it.

Keywords: home-made paocai brine, bacterial diversity, age

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1 Introduction

Fermented foods are consumed worldwide as they are important constituents of the human daily diet. In East Asia, fermented plant products are the most popular fermented food frequently consumed by people in different regions. Paocai is one of the traditional fermented vegetables in southwest China, which may date back to the third century B.C. (Liu et al., 2011). Usually, mixed, seasonal vegetable and various spices are fermented in brine with 6%-8% salt concentration (Yu et al., 2012), but it is not a sterile process and no specific inoculant is used. That is to say paocai fermentation mainly depends on the microorganisms present on the raw materials. Regarding home-made paocai, the fermentation brine is always reused and kept in the paocai jar for a long time. The microbial community of aged, home-made paocai brine (HMPB) is relatively stable and gives a particular flavor to paocai products (Xia, 2014). Some residents in southwest China have the belief that the longer the time the paocai brine is used, the better paocai brine.

The fermentation condition of paocai (e.g. room temperature and moderate salt concentration) favors the growth of lactic acid bacteria (LAB) resulting in the abundance of LAB in paocai at the end of fermentation (Chen et al., 2014). As LAB is widely considered beneficial to human health, LAB as well as the microbial community of paocai have been studied. *Lactobacillus* is the dominant genus (Yu et al., 2012) and *Pediococcus*, *Leuconostoc* and *Weissella* are often found in paocai (Chen et al., 2014). Physicochemical properties of paocai brine also impact the paocai microbial community. For instance, our previous work shows the higher acidity of paocai brine, the low bacterial diversity and the higher relative abundance of LAB (Cao et al., 2017); low salt concentration can accelerate the ripening period of paocai fermentation (Xiong et al., 2016; Zhang et al., 2016).

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Bacterial succession in the spontaneous or starter culture fermentation process of paocai has already been shown by culture-dependent methods (Xiong et al., 2012; Xiong et al., 2014). Additionally, some LAB strains from paocai have been identified and verified to have certain probiotic effects (Cao et al., 2015; Li et al., 2014). However, limited information is known about the difference in bacterial compositions and diversity of HMPB of different ages.

The Pacific Biosciences (PacBio) single-molecule, real-time (SMRT) sequencing technology can analyze the bacterial profiles of paocai brine samples based on the full-length 16S rRNA gene (Cao et al., 2017), which favors diversity analysis and affords the researcher a relatively unbiased view of microbial communities at different taxonomic levels. This study aimed to identify and compare the bacterial compositions and diversity in HMPB of different ages in order to verify whether the longer age of the brine, the better paocai brine from the view of bacterial profiles. We also compared the predicted functions of bacterial communities in different age groups of paocai brine samples.

2 Materials and methods

2.1 Samples

A total of twenty-two aged (10 + years), HMPB samples with salt concentration of 6%-8% obtained from rural families in Chongqing, China were analyzed in this work. Four paocai brine samples were sequenced in this work and the full-length 16S rRNA gene sequences of eighteen paocai brine samples were extracted from our previous projects (mgp20682 and mgp81213) in MG-RAST databases (see details in Table 2). The samples were divided into three groups based on their ages: Y10 (10-19 years), Y20 (20-29 years), and Y30 (≥30 years). The information of the samples is provided in Table 1.

Basic physicochemical properties (pH, titratable acidity (TA), and salt concentration) of the samples were measured as previously described (Cao et al., 2017).

2.2 DNA extraction

The procedures of pretreatment of paocai brine, DNA extraction, and quality examination were conducted as described previously (Cao et al., 2017). All extracted DNA samples were stored at –20°C until further analysis.

Table 1 Information about home-made paocai brine samples[a]

Sample [b]	Age, year	pH	TA, g/kg [c]**	Salt concentration, %
Y10-01	10	3.46	12.44	7.32
Y10-02	10	4.21	8.91	7.58
Y10-03	>10	3.46	12.24	7.21
Y10-04	>10	3.30	12.11	6.80
Y10-05	>10	3.50	12.19	6.66
Y10-06	>10	3.28	15.47	7.41
Y10-07	>15	3.80	10.36	7.70
Y10-08	17	3.41	8.22	7.04
Y10-09	18	3.71	10.70	7.21
Mean		3.57±0.29	11.40±2.16	7.21±0.34
Y20-01	20	3.65	14.19	6.67
Y20-02	20	3.32	15.07	7.23
Y20-03	>20	3.45	13.57	6.51
Y20-04	>20	3.50	11.32	8.02
Y20-05	>20	3.32	13.82	6.79
Y20-06	>20	3.31	15.08	6.59
Y20-07	>20	3.47	12.58	7.84
Mean		3.43±0.12	13.66±1.35	7.09±0.62
Y30-01	30	3.20	22.98	6.20
Y30-02	>30	3.26	16.19	7.68
Y30-03	>30	3.38	13.83	8.07
Y30-04	>30	3.26	14.92	7.59
Y30-05	40	3.43	16.67	6.02
Y30-06	58	3.33	15.24	6.05
Mean		3.31±0.08	16.64±3.26	6.94±0.94

Note: [a] The main components of home-made paocai brine samples were chili, ginger, radish and cowpea. [b] Y10: sample of age of 10-19 years; Y20: sample of age of 20-29 years; Y30: sample of age ≥30 years. [c] Titratable acidity expressed in grams of acid per kg of sample by multiplying a factor (0.090) appropriate to lactic acid. **: $P<0.01$.

2.3 PCR amplification and SMRT sequencing

The extracted genome was first amplified as previously described (Cao et al., 2017). The sequencing of amplicons was performed on a PacBio RS II instrument (Pacific Biosciences, USA) using P6-C4 chemistry.

2.4 Bioinformatics processing

The quality control for PCR amplifications and sequence preprocessing was conducted as previously described (Mosher et al., 2013). Sequences were further processed following the analysis pipeline as described before (Cao et al., 2017). The alpha (Shannon, Simpson, Chao1 and observed species index) and beta (the principal coordinate analysis, PCoA) diversity were evaluated.

2.5 Functional prediction

The metagenomes of paocai brine samples were predicted by the processed, full-length 16S rRNA gene sequences in PICRUSt (phylogenetic investigation of

communities by reconstruction of unobserved states, http://picrust.github.io/picrust/) (Langille et al., 2013). Amino acid sequences were translated from the predicted metagenome and aligned against the proteins/domains in the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Each protein was assigned to the KEGG orthologue group (KO) and KEGG pathway (Hou et al., 2016).

2.6 Statistical analysis

Statistical difference was tested using the R Package, Version 3.1.2 (https://www.r-project.org/). *P*-values below 0.05 between sample groups were considered statistically significant. The graph presentations were generated by the R Package, Version 3.1.2, and the Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Significantly differentiating KEGG pathways were identified according to the final reporter score calculated from the aggregated Z-score of individual KOs with the cut-off level of ≥1.6 (90% confidence according to normal distribution). Whether the significantly differentiating pathways were enriched in the Y10, Y20, or Y30 group was further determined by comparing the number of individual KOs that was enriched in each group.

2.7 Nucleotide sequence accession numbers

The sequence data reported in this study have been deposited in the MG-RAST database (Project No. mgp8012).

3 Results and discussion

3.1 Physicochemical properties of home-made brine samples

Salt concentration and raw materials impact the microbial profiles of fermented vegetable products (Jung et al., 2014). Accordingly, we selected samples with common salt concentration (6%-8%) of paocai brine and whose main materials were chili, ginger, radish and cowpea to minimize their effects on the bacterial profile. The dominant microorganisms, LAB, in paocai are sensitive to salt concentration, which further affects the production of organic acids that increase the acidity of paocai brine (Zhang et al., 2016). As shown in Table 1, the pH of paocai brine samples ranged from 3.20 to 4.21, and TA values were in the range of 8.22-22.98 g/kg. However, there was no significant correlation ($P > 0.05$) between TA and salt concentration in the samples examined, which is not consistent with our previous findings. The Kruskal-Wallis test verified that there was significant difference ($P < 0.01$) in TA among the three sample groups (TA of group Y20 and Y30 were significant higher ($P < 0.05$) than that of group Y10. This is probably due to the accumulation of more organic acid in paocai brine with age.

3.2 Sequence depth

The details of the sequencing results of individual samples in each group are shown in Table 2. The SMRT sequencing of the 16S-PCR amplicons generated a total of 134,875 raw sequence reads from the 22 HMPB samples, which were further classified into 12,764 operational taxonomic units (OTUs) under 98.65% threshold identity after quality control. Although the individual rarefaction curves (not shown) for the samples failed to reach the saturation phase, the Shannon diversity curve of most samples did reach plateau (Figure 1a). This indicated that potential phylotypes would likely to be detected along with extra sequencing, but bacterial diversity of most samples has been captured, namely the sequence depth was adequate in this study.

3.3 Bacterial diversity

The rank-abundance curve visually represents species diversity (richness and evenness) of an individual sample. The length and shape of the curve differ depending on the diversity of the sampled community and on the underlying species-abundance distribution (Lynch and Neufeld, 2015). The wider the curve, the more abundant the species composition would become. A steep gradient indicates low evenness as the high-ranking species have much higher abundances than the low-ranking species, or vice versa. As revealed in Figure 1b, although species richness and evenness differed among samples, it is clear that most samples were rich in bacterial diversity.

The α diversity is the diversity in species at individual regions or ecosystems. The bacterial α diversity of each sample was evaluated by the Shannon, Simpson, Chao 1 and observed species index (Table 2). No significant differences ($P>0.05$) were observed in the diversity estimation indicators, suggesting that the age of the aged

(10+ years) HMPB did not significantly affect the α bacterial diversity in this study. PCoA was applied to analyse the β diversity which is used to compare diversity among various communities by distance calculation between two different samples and reflect differences in microbial communities (Fang et al., 2015). The PCoA plot based on unweighted UniFrac distance of the 22 samples examined is shown in Figure 2a. The first and second principle components accounted for 10.33% and 9.53% of the variance, respectively. Although a few samples from groups Y10, Y20 and Y30 separated from each other, some overlaps existed between sample clusters from different groups. Results of the multivariate ANOVA (Figure 2b, $P>0.05$) also confirmed the differences in bacterial structure between sample groups was not significant.

Table 2 Sequence abundance and bacterial diversity in home-made paocai brine samples

Sample [a]	No. of Reads	No. of OTUs [b]	Shannon	Simpson	Chao 1	Observed species	Project number
Y10-01	4986	1171	7.07	0.95	3578.45	709.82	mgp 81213
Y10-02	3907	535	6.47	0.97	1043.07	418.49	mgp 81213
Y10-03	4660	1011	5.30	0.88	4504.61	611.93	This work
Y10-04	6399	705	6.28	0.96	976.60	416.03	mgp 81213
Y10-05	2614	890	7.97	0.98	3345.50	889.00	mgp 81213
Y10-06	7044	577	5.90	0.95	732.56	332.93	mgp 81213
Y10-07	5767	985	6.28	0.94	1950.96	561.80	mgp 81213
Y10-08	2697	624	7.07	0.98	1756.58	610.09	mgp 81213
Y10-09	4064	1266	8.04	0.99	3548.43	904.73	mgp 81213
Mean	4682±1541	863±266	6.71±0.92	0.95±0.03	2381.86±1382.54	606.09±202.46	
Y20-01	7642	928	6.70	0.97	1209.36	485.10	mgp20682
Y20-02	9435	707	5.85	0.95	752.32	343.32	mgp20682
Y20-03	8474	807	6.16	0.95	943.80	404.96	mgp20682
Y20-04	4312	564	6.50	0.96	872.91	429.86	mgp20682
Y20-05	7014	650	6.03	0.96	854.22	364.41	This work
Y20-06	7950	1006	6.77	0.97	1241.62	511.90	mgp20682
Y20-07	4977	769	4.19	0.79	3784.94	436.70	This work
Mean	7115±1855	776±154	6.03±0.88	0.94±0.06	1379.88±1076.25	425.18±60.65	
Y30-01	8088	1444	6.08	0.93	3312.34	573.70	mgp20682
Y30-02	3120	565	6.35	0.95	1755.74	497.97	mgp20682
Y30-03	10470	1650	6.82	0.95	2256.92	600.82	This work
Y30-04	6120	1024	6.82	0.97	2031.11	554.33	mgp20682
Y30-05	5881	686	6.42	0.96	1021.15	425.68	mgp20682
Y30-06	9254	1252	6.51	0.96	1642.29	528.33	mgp20682
Mean	7156±2655	1104±426	6.50±0.29	0.95±0.01	2003.26±766.15	530.14±62.33	

Note: [a] Y10: sample of age of 10-19 years; Y20: sample of age of 20-29 years; Y30: sample of age ≥30 years. [b] OUTs: Operational taxonomic units.

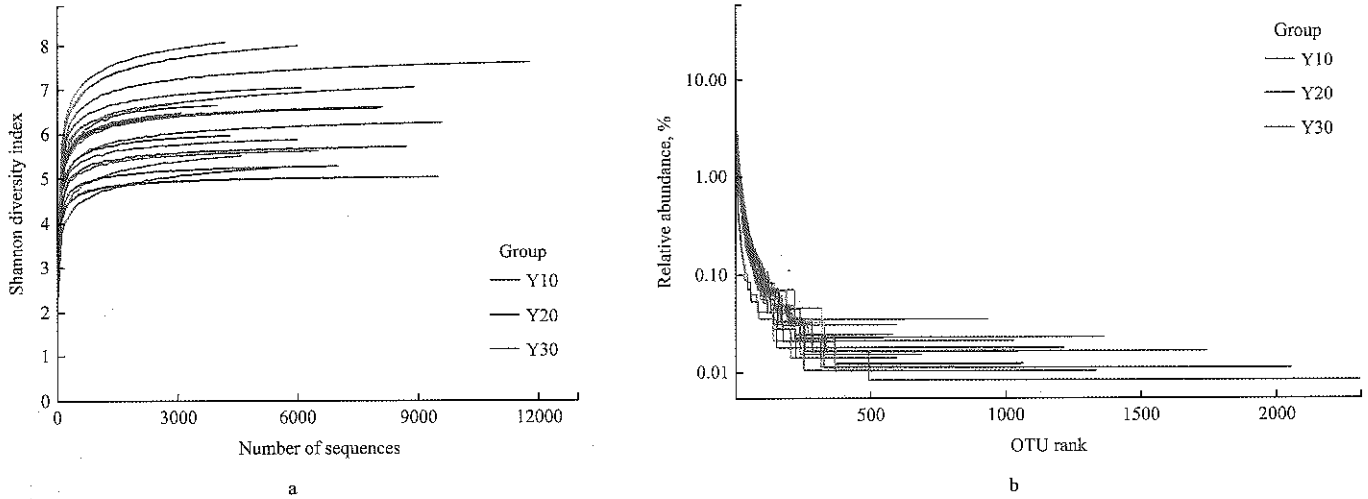

Figure 1 Shannon diversity curves (a) and rank-abundance curves (b)

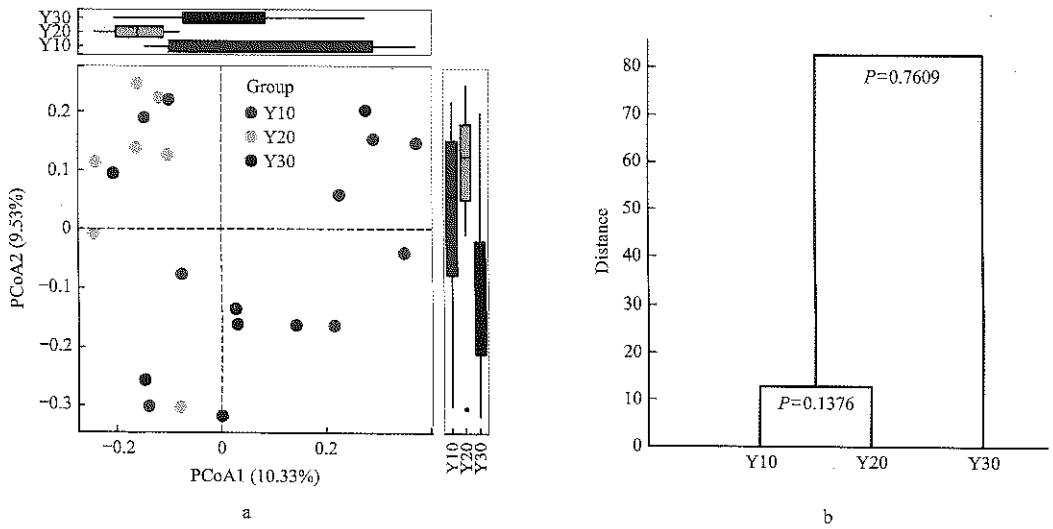

Figure 2 Principal coordinate analysis (PCoA) plots (a) and group clustering calculated with multivariate ANOVA (b) based on unweighted UniFrac distances from paocai brine samples of different age groups

3.4 Comparison of bacterial compositions between sample groups

The sequence reads were assigned to 15 bacterial phyla, 232 genera and 378 species, phyla Firmicutes and Proteobacteria, genus *Lactobacillus* (*Lb.*), and species *Lb. acetotolerans* and *Lb. brevis* were the dominant taxa in paocai brine samples (Figure 3). Other prevalent genera and species with average relative abundance over 1% are also shown in Figure 3. It is obvious that there were large variations in the relative abundance of the predominant taxa in individual sample. The results of the Kruskal-Wallis test demonstrated that there was no significant difference between different groups in these taxa (P>0.05). Opportunistic pathogen bacteria *Enterobacter cloacae*, *Kluyvera cryocrescens*, and *Serratia marcescens*, were detected in all groups. Samples Y10-01, Y20-07, Y30-01, and Y30-03 were dominated by non-LAB bacteria. In particular, Y30-01 harbored high relative abundance of the uncommon aerobic bacteria *Stenotrophomonas (Ste.) geniculate* (22.76%) and *Ste. maltophilia* (21.63%), whose relative abundance was extremely low in the other samples (not visible in Figure 3b). Moreover, *Ste. maltophilia* is a multiple-drug-resistant organism which can cause various infections in humans (Brooke, 2012), indicating the safety risk of sample Y30-01. The high abundance of health threats or undesired bacteria in some samples may be related to poor management of HMPB in some local households. These results also indicated that not the longer age of HMPB, the better bacterial profiles in it. Daily maintenance of the paocai jar and brine, such as examination of air tightness and regular utensil cleaning, is essential to keep the HMPB in a good condition. Regarding the health and safety aspects, once odd flavor or spoilage occurs in the brine, measures should be promptly taken (e.g. add spirits or use new brine).

3.5 Differential KEGG genes and pathways of bacterial community in HMPB groups of different age

When Y10 compared with Y20 group, the KOs detected in the two groups were assigned to 221 KEGG pathway, 31 pathways of which had Z value greater than 1.6; Y20 group contained most (19) enriched pathways, while only one enriched pathway in the Y10 group. When Y10 compared with Y30 group, the KOs of the two groups were assigned to 219 KEGG pathways, 30 pathways of which had Z value greater than 1.6; Y30 group also had 19 enriched pathways while Y10 group had only one. When Y20 compared Y30 group, the KOs of the two groups was also assigned to 219 KEGG pathways, 24 pathways of which had Z value greater than 1.6; Y20 group has three enriched pathways, and Y30 group had one. As shown in Table 3, the enriched pathways in the Y10 group were less than the other two groups and only D-alanine metabolism pathway was enriched in Y10 group. There were 16 pathways in both Y20 and Y30 groups more abundant than Y10 groups, which were arginine and proline metabolism, flagellum assembly, tricarboxylic acid cycle, biotin metabolism, butyric acid metabolism, lipopolysaccharide biosynthesis, sulfur repeat system, porphyrin and chlorophyll metabolism, fluorobenzoate degradation,

nitrotoluene degradation, bacterial secretion system, valine, leucine and isoleucine biosynthesis, geranium degradation, two-component system, bacterial chemotaxis and glyoxylic acid, and dicarboxylic acid metabolism.

Although the bacterial α and β diversity of Y10, Y20 and Y30 groups were not significantly different, so as the relative abundance of dominant bacteria (P<0.05), there were some differences in pathways between the age groups. By comparing the enriched pathways in the three groups, Y30 and Y20 groups had more enriched metabolic pathways than Y10 group, such as glycine, serine and threonine metabolism, arginine and proline metabolism, glyoxylic acid and dicarboxylic acid metabolism, butyric acid metabolism, tricarboxylic acid cycle, valine, leucine and isoleucine biosynthesis. These carbohydrate and amino acid metabolic pathways are likely to have an impact on the flavor of HMPB as organic acids and amino acids are essential taste compounds in the brine. These results indicated that it is likely both the longer age of HMPB samples and more carbohydrate metabolic pathways in Y20 and Y30 groups contributed to higher TA compared with Y10 group.

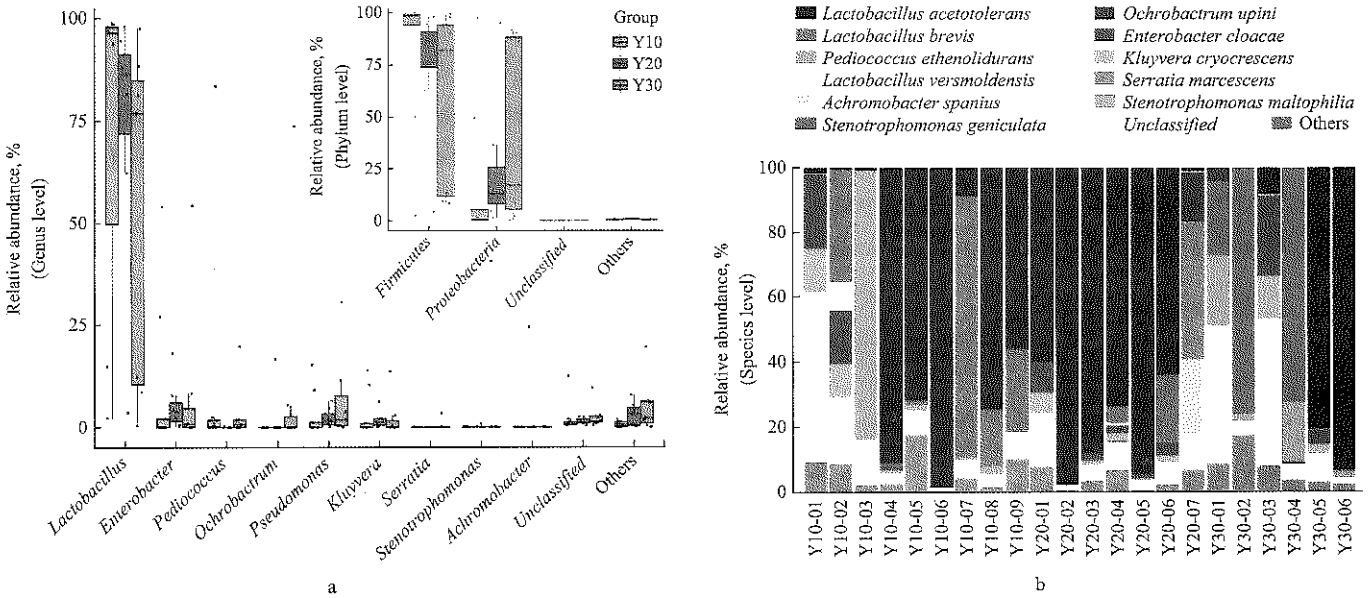

Figure 3 Bacterial compositions of different age groups of home-made paocai brine at (a) phylum and genus level, and (b) species level

Table 3 Significant differential KEGG pathway in Y10, Y20, or Y30 groups of paocai brine samples

Group	KEGG pathways	Z score	KO proportion, %	Differential pathway
				Pathways that were enriched in Y10 group
	ko00473	2.03	80.00	D-Alanine metabolism |Metabolism of other amino acids |Metabolism
				Pathways that were enriched in Y20 group
	ko00650	2.84	20.00	Butanoate metabolism |Carbohydrate metabolism |Metabolism
	ko00780	2.10	18.75	Biotin metabolism |Metabolism of cofactors and vitamins |Metabolism
	ko04122	2.51	17.65	Sulfur relay system |Folding, sorting and degradation |Genetic Information Processing
	ko00330	2.56	17.58	Arginine and proline metabolism |Amino acid metabolism |Metabolism
	ko00020	2.51	17.07	Citrate cycle (TCA cycle) |Carbohydrate metabolism |Metabolism
	ko00195	4.82	15.38	Photosynthesis |Energy metabolism |Metabolism
	ko03070	7.30	12.68	Bacterial secretion system |Membrane transport |Environmental Information Processing
Y10 vs Y20	ko00630	3.18	12.68	Glyoxylate and dicarboxylate metabolism |Carbohydrate metabolism |Metabolism
	ko02020	7.95	12.42	Two-component system |Signal transduction |Environmental Information Processing
	ko00910	2.41	7.50	Nitrogen metabolism |Energy metabolism |Metabolism
	ko00540	3.01	6.06	Lipopolysaccharide biosynthesis |Glycan biosynthesis and metabolism |Metabolism
	ko00860	3.28	5.19	Porphyrin and chlorophyll metabolism |Metabolism of cofactors and vitamins |Metabolism
	ko02030	4.58	3.85	Bacterial chemotaxis |Cell motility |Cellular Processes
	ko02040	5.44	0.00	Flagellar assembly |Cell motility |Cellular Processes
	ko00633	3.10	0.00	Nitrotoluene degradation |Xenobiotics biodegradation and metabolism |Metabolism
	ko00281	2.75	0.00	Geraniol degradation |Metabolism of terpenoids and polyketides |Metabolism
	ko00290	2.69	0.00	Valine, leucine and isoleucine biosynthesis |Amino acid metabolism |Metabolism
	ko00364	1.92	0.00	Fluorobenzoate degradation |Xenobiotics biodegradation and metabolism |Metabolism

Group	KEGG pathways	Z score	KO proportion, %	Differential pathway
Y10 vs Y30				Pathways that were enriched in Y10 group
	ko00473	2.20	80.00	D-Alanine metabolism |Metabolism of other amino acids |Metabolism
				Pathways that were enriched in Y30 group
	ko00260	2.00	18.03	Glycine, serine and threonine metabolism |Amino acid metabolism |Metabolism
	ko04122	2.61	17.65	Sulfur relay system |Folding, sorting and degradation |Genetic Information Processing
	ko00720	1.84	16.42	Carbon fixation pathways in prokaryotes |Energy metabolism |Metabolism
	ko00330	3.07	15.22	Arginine and proline metabolism |Amino acid metabolism |Metabolism
	ko00630	3.65	14.08	Glyoxylate and dicarboxylate metabolism |Carbohydrate metabolism |Metabolism
	ko00650	3.05	13.56	Butanoate metabolism |Carbohydrate metabolism |Metabolism
	ko03070	7.72	11.27	Bacterial secretion system |Membrane transport |Environmental Information Processing
	ko00860	3.31	10.39	Porphyrin and chlorophyll metabolism |Metabolism of cofactors and vitamins |Metabolism
	ko02020	7.22	10.09	Two-component system |Signal transduction |Environmental Information Processing
	ko00020	3.08	9.76	Citrate cycle (TCA cycle) |Carbohydrate metabolism |Metabolism
	ko00364	1.61	8.33	Fluorobenzoate degradation |Xenobiotics biodegradation and metabolism |Metabolism
	ko00290	2.93	6.67	Valine, leucine and isoleucine biosynthesis |Amino acid metabolism |Metabolism
	ko00540	3.89	6.06	Lipopolysaccharide biosynthesis |Glycan biosynthesis and metabolism |Metabolism
	ko02030	5.06	3.85	Bacterial chemotaxis |Cell motility |Cellular Processes
	ko02040	5.56	0.00	Flagellar assembly |Cell motility |Cellular Processes
	ko00281	2.83	0.00	Geraniol degradation |Metabolism of terpenoids and polyketides |Metabolism
	ko00633	2.43	0.00	Nitrotoluene degradation |Xenobiotics biodegradation and metabolism |Metabolism
	ko00780	2.20	0.00	Biotin metabolism |Metabolism of cofactors and vitamins |Metabolism
Y20 vs Y30				Pathways that were enriched in Y20 group
	ko02060	5.88	91.30	Phosphotransferase system (PTS) |Membrane transport |Environmental Information Processing
	ko00195	5.04	84.62	Photosynthesis |Energy metabolism |Metabolism
	ko00051	2.38	83.08	Fructose and mannose metabolism |Carbohydrate metabolism |Metabolism
				Pathways that were enriched in Y30 group
	ko02040	5.28	16.67	Flagellar assembly |Cell motility |Cellular Processes

Note: the table only includes pathways that have a high (≥80%) or a low (≤20%) proportion of KO of gene abundance, indicating that these pathways are either enriched in the former or latter sample group, respectively.

4 Conclusion

To our knowledge, this is the first report on the comparison of bacterial diversity in home-made paocai brine of different age (10+ years) based on SMRT sequencing. Our results revealed that for aged, home-made paocai brine, the bacterial diversity or compositions may not be classified by age. But home-made paocai brine with longer age likely have more bacterial metabolic pathways which may affect the paocai flavour.

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