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Correlation analysis between amino acids and bacterial communities of *Wuliangye*-flavour liquor fermentation in aged fermentation pit

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Abstract

Fermented grain (*Zaopei*) is the main microbial habitat and biochemical reaction system of health factors for *Wuliangye*-flavour liquor fermentation. In the present work, the bacterial communities in four depths of *Zaopei*, from the same fermentation pit, aged over 60 years, and amino acids as major health factors in four liquors from directly corresponding *Zaopei* were investigated by Illumina MiSeq sequencing and liquid chromatography mass spectrometry (LC-MS), respectively. Results showed that a total of 18 amino acids were detected in the four liquors, and eight dominant bacterial genera were observed in four *Zaopei* corresponding to the four liquors. Meanwhile, total amino acids, 12 amino acids (Glu, Asp, Val, Ile, Cys, Met, Lys, Arg, Gly, Ala, Tyr, and Thr), bacterial richness, and the percentages of *Lactobacillus* and *Pseudomonas* increased with the increase in *Zaopei*'s depth; five amino acids (Cit, Phe, Leu, Pro, and Ser), and the percentages of *Pediococcus* and *Bacteroides*, first increased and then decreased, with the increase in *Zaopei*'s depth. Moreover, the 12 amino acids were significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) positively correlated with *Lactobacillus* and *Pseudomonas*. Therefore, these results can provide relevant data support for increasing amino acid content in *Wuliangye*-flavour liquor.

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Introduction

Researchers are nowadays focused on the importance of bioactive compounds in foods; and foods with bioactive compounds are often referred to as healthy foods (Banwo *et al.*, 2021). The interest of consumers is the consumption of healthy food, whereas the interest of food manufacturers is that consumers recognise the produced "healthier" food items on the shelves, so they can satisfy their demands (Plasek *et al.*, 2020). Food environment can influence opportunities and barriers to food access (Costa *et al.*, 2019). At present, consumers are widely interested on the nutrition and health status of Chinese white liquor (*baijiu*) (Huo *et al.*, 2020), with many bioactive compounds in *baijiu* have been found to be beneficial to human health such as amino acids, phenols, acids, pyrazine, and peptides, which have the functions of promoting ethanol metabolism, improving comfort after drinking, anti-oxidation, anti-inflammatory, anti-cancer, and prevention and treatment of cardiovascular diseases (Wu *et al.*, 2017; Liu *et al.*, 2020).

Wuliangye-flavour liquor is one of the famous *baijiu* in China, and its consumption has ranked among the top three in recent years (Kim, 2009; Fan *et al.*, 2021; Wang *et al.*, 2021). Under the action of microorganisms, five fermented grains (sorghum, glutinous rice, rice, wheat, and maize) namely *Zaopei*, can be fermented in a fermentation pit into *Wuliangye*-flavour liquor after 70-160 days (Zheng *et al.*, 2018; You *et al.*, 2021). Since the fermentation pit is one of the necessary facilities for *Wuliangye*-flavour liquor fermentation, and that the pit must be continuously used for over 10 years to produce high quality *Wuliangye*-flavour liquor (Zhang *et al.*, 2015), this suggests that the microorganisms in the pit play a key role in liquor fermentation (Zhang *et al.*, 2015). Therefore, it is necessary to enrich the microorganisms in *Wuliangye*-flavour liquor

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fermentation by establishing a high-throughput screening method based on health factors, and further investigate the fermentation characteristics and conditions of the microorganisms.

Certain bacterial genera such as *Lactobacillus* and *Bacillus* contribute to promoting the formation of *baijiu*'s quality and health factors during fermentation (Zhang *et al.,* 2015). In addition, *Lactobacillus* can provide other microorganisms with amino acids and vitamins for their reproduction and growth (Xie *et al.,* 2008), while *Bacillus* can increase the production of tetramethylpyrazine and provide the possibility for enriching the tetramethylpyrazine in *baijiu* (Xu *et al.*, 2018). Therefore, the main objective of the present work was to investigate the amino acids as major health factors in four different liquors, from the same fermentation pit, aged over 60 years, by liquid chromatography mass spectrometry (LC-MS), and bacterial communities in four *Zaopei* corresponding to the four liquors by Illumina MiSeq sequencing. Furthermore, the multivariate statistical techniques were used to investigate amino acids, bacterial communities, and their correlations during *Wuliangye*-flavour liquor fermentation to offer a guidance for increasing the amino acid content in *Wuliangye*-flavour liquor.

Materials and methods

Materials

Four liquors and their corresponding *Zaopei* were simultaneously collected in the same fermentation pit (same batch), aged over 60 years, from a *Wuliangye*-flavour liquor company in Yibin, Sichuan, China (May 2020). Four *Zaopei* were respectively taken from the bottom layer (BO), middle layer (MI), upper layer (UP), and top layer (TO) of the fermentation pit, as described previously (Wang *et al.*, 2021). The sampling method of *Zaopei* was carried out according to the 5-point sampling method (Wang *et al.*, 2021), and then, the samples were respectively placed into the sterilising bag with a mark, and stored at -80°C for amplicon sequencing analysis. Meanwhile, four liquors corresponding to the four *Zaopei* were both collected from the part, with distillation time of 0.5 - 10.0 min.

Citric acid, sodium citrate, chloroform, acetone, toluene, acetic acid, potassium hydroxide, trichloroacetic acid, methanol, acetonitrile, and tetrahydrofuran were purchased from Kelon Chemical Reagent Factory, Chengdu, China. The standards of glutamic acid (Glu), aspartic acid (Asp), citrulline (Cit), threonine (Thr), glycine (Gly), arginine (Arg), serine (Ser), methionine (Met), leucine (Leu), proline (Pro), isoleucine (Ile), alanine (Ala), tyrosine (Tyr), cysteine (Cys), valine (Val), histidine (His), phenylalanine (Phe), lysine (Lys), *o*-phthalaldehyde (OPA), 9-fluorenylmethylchloroformate (FMOC), and triethylamine (chromatographic pure) were purchased from Sigma-Aldrich (Shanghai, China). The Fast DNA SPIN Kit for Soil (MP Biomedicals, OH, USA) was used following the manufacturer's instruction to extract DNA from pit mud samples.

Identification and quantification of amino acids in liquors

Precisely 1 mL of *Wuliangye*-flavour liquor was mixed with 10% trichloroacetic acid solution in equal volume, and centrifuged at 10,000 rpm for 15 min. Following filtration by aqueous phase filter membrane (0.45 μm), 500 μL of sample were tested. The amino acids in liquors were determined by OPA-FMOC pre-column derivatisation, while the retention time was used for qualitative analysis, and the peak area was quantified by external standard method (Wang *et al.*, 2021).

Illumina MiSeq sequencing for bacterial communities in Zaopei

To analyse the taxonomic composition of the bacterial communities in *Zaopei*, the universal primer pairs, 515F and 806R, which incorporated Illumina adapters and barcode sequences, were used to amplify the V4 hypervariable region of the 16S rRNA gene, using a two-step amplification procedure. DNA extraction, polymerase chain reaction (PCR), and Illumina MiSeq sequencing (2-by 150-bp reads) were performed by Wuhan Biotechnology Co., Ltd. (Wuhan, China), as previously described (Huang *et al.*, 2021). Each sample was extracted twice, and each extraction was analysed three times. Data processing was performed mainly using QIIME (Quantitative Insights Into Microbiota, V1.8.0); chimeric sequences were excluded with default parameters, and sequences with similarities > 97% were clustered into one operational taxonomic unit (OTU) using QIIME. The taxonomical assignment of each OTU was performed using the Greengenes database (http://greengenes.secondgenome.com) at a 90% confidence level (Li *et al.*, 2016; Wang *et al.*, 2018). After calculating the OTU matrix, statistical analysis

was applied using alpha indices (Shannon, Simpson, Chao1, and ACE) which were calculated using QIIME.

Statistical analysis

One-way analysis of variance (ANOVA) with the least-significant difference (LSD) method ($p < 0.05$) was applied to compare the alpha indices between different *Zaopei* and the contents of amino acids in liquors. Principal component analysis (PCA) and cluster analysis were performed using SIMCA 14.1 software and R Project 3.5, respectively. Linear discriminant analysis effect size (LEfSe) algorithm was performed using the online interface utilising the Huttenhower Lab Galaxy server. Pearson correlation analysis was performed using SPSS 22.0 software, and visual network analysis was performed using Cytoscape 3.7 software.

Results and discussion

Amino acids in four liquors

Eight essential amino acids (Thr, Met, Leu, Ile, Val, His, Phe, and Lys) and 10 non-essential amino acids (Glu, Asp, Cit, Gly, Arg, Ser, Pro, Ala, Tyr, and Cys) were detected in the four liquors. Meanwhile, PCA showed that the amino acids of liquors in the same fermentation pit changed significantly, and four clusters were respectively formed (Figure 1A).

The contents of 12 amino acids (Glu, Asp, Val, Ile, Cys, Met, Lys, Arg, Gly, Ala, Tyr, and Thr) increased significantly with the *Zaopei* from top layer to bottom layer ($p < 0.05$); the contents of five amino acids (Cit, Phe, Leu, Pro, and Ser) first increased and then decreased, with the increase in *Zaopei*'s depth; of them, the contents of Cit, Phe, Leu, and Pro in TO were the lowest ($p < 0.05$); and His was only detected in TO (Figure 1B). Therefore, Glu, Asp, Val, Ile, Cys, Met, Lys, Arg, Gly, Ala, Tyr, and Thr were classified in one group; Cit, Phe, Leu, and Pro were classified in one group; and Ser and His were divided into different groups (Figure 1B). The total amino acid contents in TO, UP, MI, and BO were 23.42, 37.71, 45.55, and 48.75 mg/L, respectively.

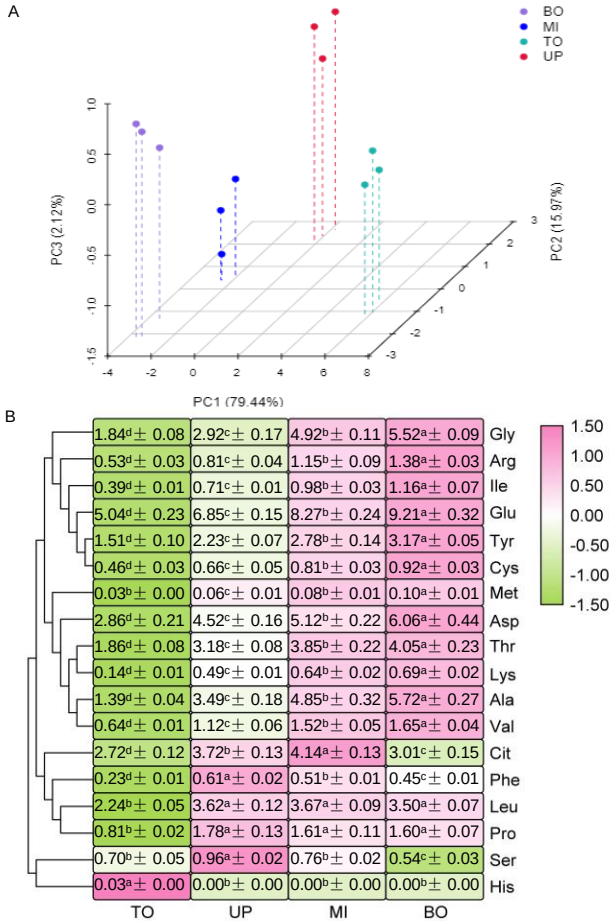

Figure 1. PCA **(A)** and cluster analysis **(B)** of amino acids in four liquors. Data are mean ± standard deviation of triplicates ($n = 3$). Means with different lowercase superscripts within a row are significantly different ($p < 0.05$).

Bacterial communities in four Zaopei

After filtering out the low-quality reads and chimeras, 21,673 to 39,570 sequences were obtained, and 296 to 415 OTUs were generated. These OTUs were classified into 40 phyla, including 37 bacterial and three archaeal phyla. With the increase in *Zaopei*'s depth, the bacterial richness and diversity also statistically increased ($p < 0.05$, Table 1).

Table 1. Microbial diversity indices calculated based on the cut-off of 97% identity of 16S rRNA gene region.

Depth	Community richness		Community diversity	
	ACE	Chao1	Shannon	Simpson
TO	2153.46 ± 87.13^{d}	2201.42 ± 76.11^{d}	5.46 ± 0.14^{d}	0.69 ± 0.01^{d}
UP	2469.11 ± 93.52^{c}	2560.75 ± 83.69^{c}	6.67 ± 0.19^{c}	0.78 ± 0.02^{c}
MI	3370.53 ± 126.24^{b}	3537.68 ± 103.68^{b}	7.96 ± 0.18^{b}	0.83 ± 0.02^{b}
BO	3931.07 ± 132.12^{a}	4257.56 ± 122.37^{a}	8.48 ± 0.20^{a}	0.91 ± 0.02^{a}

Data are mean ± standard deviation of triplicates ($n = 3$). Means followed by different lowercase superscripts within a column are significantly different ($p < 0.05$). TO: top layer, UP: upper layer, MI: middle layer, and BO: bottom layer.

Due to the changes in environmental conditions (temperature, humidity, oxygen content, and pH) in the fermentation pit (Tao *et al.*, 2014; Hu *et al.*, 2016), the bacterial communities of *Zaopei* changed dynamically with the increase in depth (Figure 2). For instance, the dominant bacteria (genus level) in TO were *Lactobacillus* (36.83%), *Bacillus* (22.35%), and *Pediococcus* (12.17%); *Lactobacillus* (39.36%), *Pediococcus* (23.50%), and *Bacillus* (10.14%) were the dominant bacteria in UP; the dominant bacteria in MI were *Lactobacillus* (45.91%), *Pediococcus* (20.74%), and *Ochrobactrum* (8.40%); and the dominant bacteria in BO (50.64% *Lactobacillus*, 13.92% *Pediococcus*, and 7.64% *Bacillus*) were consistent with UP (Figure 2). Meanwhile, the percentages of *Lactobacillus* and *Pseudomonas* increased with the increase in *Zaopei*'s depth; the percentages of *Clostridium* showed the opposite trend; and the percentages of *Pediococcus* and *Bacteroides* first increased and then decreased with the increase in *Zaopei*'s depth (Figure 2). *Lactobacillus*, being anaerobic bacteria and preferring acidic environment (Yang *et al.*, 2020; Da Silva *et al.*, 2021), significantly ($p < 0.05$) increased due to the content of oxygen and level of pH decreasing with the increase in *Zaopei*'s depth (Zhao *et al.*, 2020).

Through LEfSe, it was found that the percentages of *Bacillus* and *Clostridium* in TO were significantly higher ($p < 0.05$) than that in other samples; the percentages of *Bacteroides* and *Acinetobacter* in UP increased significantly ($p < 0.05$); the percentages of *Pediococcus* increased significantly ($p < 0.05$) in MI; and the percentages of *Lactobacillus*, *Pseudomonas*, *Ochrobactrum*, *Paludibacter*, and *Methanosarcina* in BO were significantly higher ($p < 0.05$) than that in the other samples (Figure 3).

Correlation analysis between amino acids and bacterial communities

The correlation between 18 amino acids and eight dominant bacteria was determined by Pearson correlation analysis, aiming to obtain more useful information by clarifying their relationship. As showed in Figure 4, 12 amino acids (Glu, Asp, Val, Ile, Cys, Met, Lys, Arg, Gly, Ala, Tyr, and Thr) were significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) positively correlated with *Lactobacillus* and *Pseudomonas* (red edges); Cit was significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) positively correlated with *Pediococcus*, but significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) negatively correlated with *Bacillus* and *Proteiniclasticum* (green edges). Meanwhile, Leu and Phe were significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) positively correlated with *Bacteroides*, but significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) negatively correlated with *Clostridium*.

Therefore, *Lactobacillus* and *Pseudomonas* can be beneficial to form Glu, Asp, Val, Ile, Cys, Met, Lys, Arg, Gly, Ala, Tyr and Thr; and this result verified that *Lactobacillus* had positive relationships with the quality of *Wuliangye*-flavour liquor, which is a strong-flavour liquor (Yang *et al.*, 2017; Liu *et al.*, 2019; Wang *et al.*, 2019).

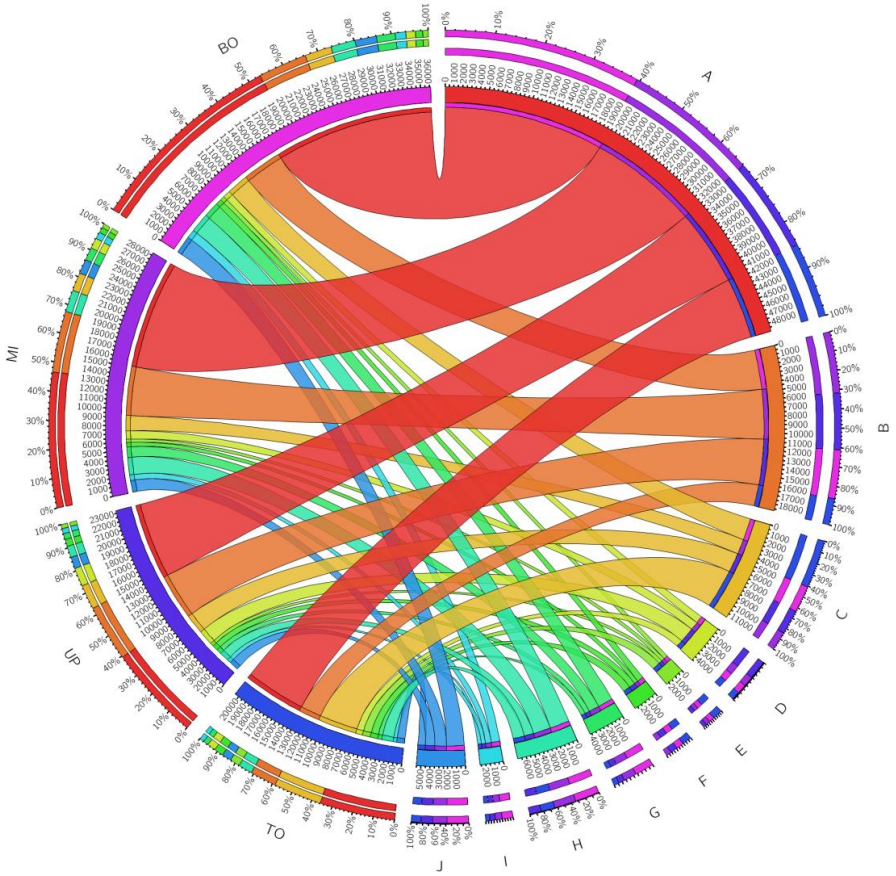

Figure 2. Bacterial communities in four *Zaopei* at the genus level. **(A)**: *Lactobacillus*, **(B)**: *Pediococcus*, **(C)**: *Bacillus*, **(D)**: *Bacteroides*, **(E)**: *Clostridium*, **(F)**: *Proteiniclasticum*, **(G)**: *Pseudomonas*, **(H)**: *Ochrobactrum*, **(I)**: unclassified, **(J)**: others, **(TO)**: top layer, **(UP)**: upper layer, **(MI)**: middle layer, and **(BO)**: bottom layer.

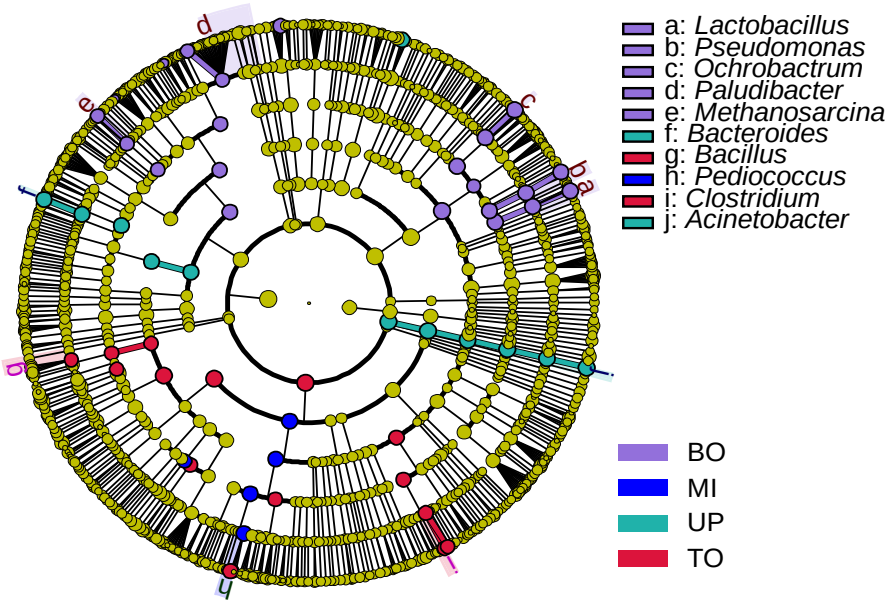

Figure 3. Linear discriminant analysis effect size (LEfSe) of bacterial communities in *Zaopei*. BO: bottom layer, MI: middle layer, UP: upper layer, and TO: top layer.

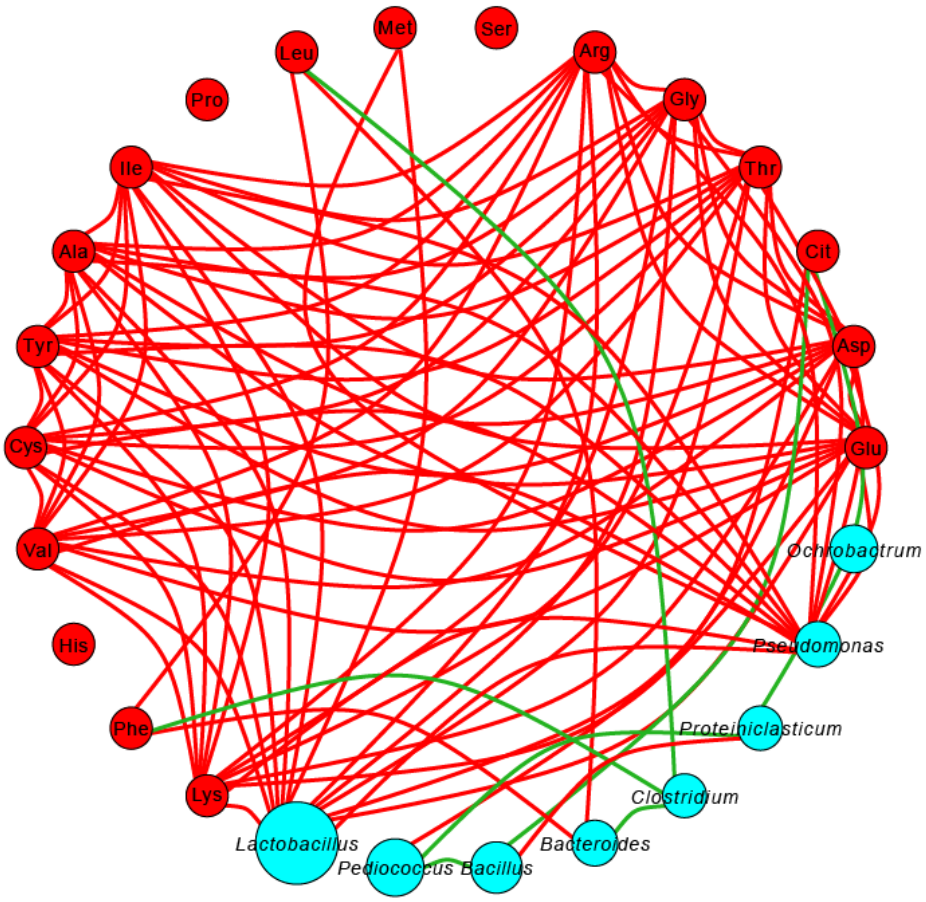

Figure 4. Interaction of bacteria and amino acids in four samples on the basis of the Pearson correlation analysis. The connection indicates a statistically significant ($p < 0.01$) strongly positive (red line) or negative (green line) correlation with Spearman's $|\rho| > 0.8$.

Conclusion

A total of 18 amino acids were detected in four liquors, and eight dominant bacterial genera were observed in four *Zaopei* corresponding to the four liquors in a *Wuliangye*-flavour liquor fermentation, pit aged over 60 years. In summary, Glu, Asp, Val, Ile, Cys, Met, Lys, Arg, Gly, Ala, Tyr, and Thr, bacterial richness, and the percentages of *Lactobacillus* and *Pseudomonas* increased with the increase in *Zaopei*'s depth; Cit, Phe, Leu, Pro, and Ser, and the percentages of *Pediococcus* and *Bacteroides* first increased and then decreased with the increase in *Zaopei*'s depth. The 12 amino acids were significantly ($p < 0.01$) and strongly ($|\rho| > 0.8$) positively correlated with *Lactobacillus* and *Pseudomonas*. Therefore, these results can be used as reference to establish the relationship between amino acids and bacteria during *Wuliangye*-flavour liquor fermentation, and provide relevant data support for increasing amino acid content in *Wuliangye*-flavour liquor.

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