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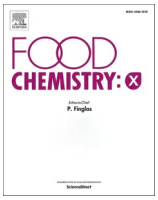

Characterization and correlation analysis of microbial flora and flavor profile of stinky acid, a Chinese traditional fermented condiment

Zhendong Zhang [a], Huijun Zhao [b,c], Renzhi Zhu [a], Shaojing Cheng [a], Yuanqi Yu [a], Lan Xiang [a], Zhipan Xiang [a], Zhuang Guo [b,c], Yurong Wang [b,c,*]

[a] School of Biological Science and Agriculture, Qiannan Normal University for Nationalities, Duyu, Guizhou Province, China
[b] Hubei Provincial Engineering and Technology Research Center for Food Ingredients, Hubei University of Arts and Science, Xiangyang, Hubei Province, China
[c] Xiangyang Lactic Acid Bacteria Biotechnology and Engineering Key Laboratory, Hubei University of Arts and Science, Xiangyang, Hubei Province, China

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ABSTRACT

To explore the microbial diversity and flavor profiles of stinky acid, we utilized high-throughput sequencing, culture-based techniques, and bionic E-sensory technologies. The results revealed a significant correlation between the acidity levels of stinky acid and the richness of its microbial community. Ten core bacterial genera and three core fungal genera exhibited ubiquity across all stinky acid samples. Through E-nose analysis, it was found that sulfides constituted the principal odor compounds responsible for stinky acid's distinct aroma. Further insights arose from the correlation analysis, indicating the potential contribution of *Debaryomyces* yeast to the sour taste profile. Meanwhile, three genera—*Rhizopus* and *Thermoascus* and *Companilactobacillus*—were identified as contributors to aromatic constituents. Interestingly, the findings indicated that *Rhizopus* and *Thermoascus* could reduce the intensity of the pungent odor of stinky acid. In summary, this investigation's outcomes offer new insights into the complex bacterial diversity of stinky acid.

1. Introduction

China boasts a diverse array of traditional fermented foods, many characterized by their distinctive stinky aromas. Notable examples include stinky tofu (also known as gray sufu), Yan-dong-gua (naturally fermented wax gourd), and Chouguiyu (fermented mandarin fish with a pungent odor) (Song et al., 2021; Wang et al., 2021; Wu et al., 2016). Particularly in Guizhou Province, located in China's southern region, the well-known fermented food product "stinky acid" (Chousuan) is notably prominent. This fermented stinky acid is distinguished as one of the trios of renowned fermented sour foods in Dushan, including shrimp-acid, stinky-acid, and yan-acid (Xu et al., 2020). Stinky acid exhibits a unique and discernible malodorous characteristic, distinguishing it from other fermented foods. Despite its odor often being met with aversion, it possesses an exquisite taste relished by the local populace. Served as a condiment, stinky acid is used in preparing meat and tofu (bean curd) dishes, as well as hot pot. Predominantly popular in the south of Guizhou Province, stinky acid has garnered favor among locals (Wei et al., 2022).

Stinky acid, a solid fermented delicacy, is crafted using local traditional production methods (Xu et al., 2020). These methods involve utilizing raw materials, including impatiens (*Impatiens balsamina* L.), primary green vegetables (*Brassica juncea* L.), indigenous pungent litse fruit (*Litsea pungens* Hemsl.), bamboo shoots, and fried wheat. The initial steps involve washing, draining, and segmenting impatiens and green vegetables. Subsequently, the segmented raw materials and other ingredients, including chopped bamboo shoots, fried wheat, and pungent litse fruit, are meticulously arranged in specially designed pottery jars resembling pickle jars. These contents are then compacted in the jars. Finally, the pottery jars are hermetically sealed and placed in the shade to initiate a natural fermentation process lasting approximately 1 month (Xu et al., 2020). It is noteworthy that this fermentation occurs without the introduction of a starter culture. The final product exhibits a verdant hue and a consistency reminiscent of sauce. The specific ratio of raw materials may vary slightly depending on the maker's preferences. Thus, stinky acid distinctly differs from other stinky foods in both its

Abbreviations: LAB, Lactic acid bacteria; OTU, operational taxonomic unit; PERMANOVA, permutational multivariate analysis of variance; MRS, de Man, Rogosa, and Sharpe; PDA, potato dextrose agar; ITS, internal transcribed spacer.

* Corresponding author at: Hubei Provincial Engineering and Technology Research Center for Food Ingredients, Hubei University of Arts and Science, Xiangyang, Hubei Province, China.
E-mail address: wang_HFu6456@163.com (Y. Wang).

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production process and the use of raw materials (Song et al., 2021; Wang et al., 2021; Wu et al., 2016). Furthermore, a natural fermentation approach that omits starter cultures and the unsterilized raw materials and pottery jars in the fermentation process prevent precise control over the final product's microbial composition, made the microbial composition in traditional food extremely complicated (Bassi et al., 2015; K. Zhang et al., 2023). Overall, stinky acid significantly differs from other stinky foods in terms of production process, environmental factors, and raw materials used. This may lead to a unique microbial flora in stinky acid. In recent years, despite growing interest in stinky acid's unique flavor, there have been few studies focusing on its microbial diversity and the central microbial community.

Flavor is indeed one of the most significant characteristics of food, influencing consumer decision-making, and the distinctive flavor profiles are largely contributed to by microbial consortia (Chen et al., 2021; Sequino et al., 2022). The relationship between microorganisms and food flavor characteristics in certain foods has been established. Wang et al. demonstrated that *Arcobacter*, *Psychrilyobacter*, and *Shewanella* are pivotal in conferring characteristic flavors to Chouguiyu (Wang et al., 2021). Similarly, Song et al. used high-throughput sequencing and HPLC-based metabolite analysis to show that *Fusobacterium*, *Providencia*, *Lactobacillus*, and *Bacillus* could reduce propionic acid content in stinky sufu (Song et al., 2021). Moreover, microbial metabolic network analysis identified *Lactobacillus* and *Dekkera* as significant flavor-producing microbes (Xia et al., 2021). Stinky acid, a fermented food from the south of Guizhou Province, China, is not widely accepted outside this area due to its unique odor. Despite the significant role of microbial communities in the fermentation process, the flavor characteristics of stinky acid and their underlying microbial interactions remain largely unexplored. This understanding would offer insights into the fermentation process and provides a foundation for harnessing these microorganisms to optimize the aroma of stinky acid.

Thus, this study aimed to analyze the flavor characteristics and microbial community profile of stinky acid. Furthermore, it aimed to identify the correlation between the flavor characteristics and the dominant microbial genera. Specifically, 42 representative samples of stinky acid were collected. Initially, the counts of bacteria, lactic acid bacteria (LAB), yeast, and molds were detected using culture-based technology, alongside the determination of pH and total acidity. Subsequently, microbial community profiles, including bacterial and fungal flora, were analyzed using high-throughput sequencing. Finally, flavor profiles were evaluated with E-tongue and E-nose sensors. The results of this study are expected to significantly enhance our understanding of microbial diversity, the relationship between functional species and pathways, and provide substantial data support for the production and quality improvement of stinky acid.

2. Materials and methods

2.1. Sampling

In February 2023, a study on the microbial diversity of stinky acid was conducted in Dushan County (107°41′-107°55′ E, 25°04′-25°31′ N), located in the Qiannan Buyi and Miao Autonomous Prefecture, Guizhou Province, China. A total of 42 stinky acid samples were collected from local agricultural markets, labeled CS01 to CS42. All samples were prepared using natural fermentation techniques, with similar raw materials (impatiens, green vegetables, bamboo shoots, pungent litse fruit, and fried wheat), and comparable processing steps. Approximately 150 g of each sample was preserved in duplicate in sterile containers with screw caps at 4℃. Upon arrival at the laboratory, one set of duplicates was immediately stored at −80℃ for amplicon sequencing. The other set was immediately allocated for other analyses.

2.2. Microbiological and physicochemical analysis

Microbe quantification followed the method described by Zheng et al. (Zheng et al., 2012). Specifically, the number of total aerobic bacteria, LAB, and yeast was evaluated using plate count agar (Hepebio, Qingdao, China), MRS agar (formulated with 1 % calcium carbonate, Hepebio, Qingdao, China) (De Man et al., 1960), and potato dextrose agar (PDA, Hepebio, Qingdao, China), respectively. For total aerobic bacteria and LAB enumeration, culture plates were incubated at 30 °C for 3–5 days. However, yeast quantification required incubation at 25 °C. MRS agar, crucial for LAB cultivation, was incubated in an anaerobic environment in a DG250 workstation (Don Whitley Scientific Ltd., Shipley, Yorkshire, UK). This anaerobic condition was achieved by infusing the workstation with a gas mixture of 85 % nitrogen, 10 % carbon dioxide, and 5 % hydrogen. In contrast, plate count and PDA agars were incubated under aerobic conditions.

The water content of stinky acid was assessed using the direct drying method, according to the GB 5009.3–2016 standard. Total acid content quantification followed the GB 5009.3–2016 guidelines. Additionally, pH was measured according to GB 5009.237–2016 using a PB-10 pH meter (Sartorius, Germany).

2.3. Identification of LAB isolates

Isolation of LAB strains followed the procedures detailed by Zhang et al. (2023). Each stinky acid sample underwent 10-fold serial dilution to achieve a 10^{-6} dilution. Subsequently, dilutions from 10^{-2} to 10^{-6} were uniformly spread onto MRS plates supplemented with 1 % calcium carbonate. These plates were incubated at 30 °C in an anaerobic environment for 3–5 days. Colonies exhibiting a transparent halo were selected for purification and preserved at −80 °C. The preservation medium was MRS broth with 25 % glycerol.

Candidate LAB isolates were identified through 16S rDNA sequencing, following the workflow described by Zhang et al. (2019). First, total DNA was extracted using an E.Z.N.A.@Bacterial DNA Kit (Omega Bio-tek Inc., Norcross, GA, USA). The 16S rRNA gene was amplified with universal primers 27F and 1492R, using the extracted DNA as a template, as described by Osborne et al. (2005). The PCR products were then cloned into the pMD18-T vector (TaKaRa, Dalian, China), and used to transform DH5-*α Escherichia coli* cells. Positive clones were selected and forwarded to Shanghai Sunny Biotechnology Co., Ltd.

The 16S rRNA sequences obtained were used to conduct a Blastn search in the EzBiocloud database (Yoon et al., 2017). The isolates' 16S rRNA sequences were aligned with closely matched sequences from the EzBiocloud database. The sequences were used to construct a neighbor-joining phylogenetic tree with Mega X software for taxonomic classification (Kumar et al., 2018).

2.4. DNA extraction and high-throughput sequencing

To explore stinky acid's microbial diversity, metagenomic DNA was extracted and preserved at −80 °C. The 16S rRNA V_3-V_4 and ITS regions were selected for PCR amplification, followed by MiSeq sequencing. Metagenomic DNA from stinky acid samples was extracted using an E.Z.N.A.® Food DNA Kit, with DNA quality verified via a Nanodrop ND-2000C microUV spectrophotometer (Thermo Scientific, Wilmington DE, USA), following the manufacturer's guidelines.

For bacterial diversity examination, the 16S rRNA V3-V4 region was amplified with universal primers 338F and 806R, as noted by Wang et al. (2016). For fungal diversity assessment, universal primers ITS3 F and ITS4 R were used, following Kobayashi et al. (2011). PCR products from these primers were used to construct sequencing libraries. Sequencing was performed with the paired-end method on a HiSeq 4000 system at Majorbio Biotech Co., Ltd. (Beijing, China). Ultimately, sequencing was conducted using the paired-end method on the HiSeq 4000 system

(Illumina, CA, USA) at Majorbio Biotech Co., Ltd. (Beijing, China). During sequencing, 150-bp reads were generated in both directions.

2.5. Bioinformatic and statistical analysis

To examine microbial diversity, raw sequencing data were processed with QIIME 1.9.0 (Caporaso et al., 2010). Initially, paired-end raw read data were filtered through TrimGalore v0.6.6 (Krueger, 2015). This process yielded high-quality reads, subsequently merged using FLASH (Magoč et al., 2011). Subsequently, potential chimera sequences were identified and removed using ChimeraSlayer on QIIME (Haas et al., 2011). The remaining sequences were clustered into OTUs based on a 97 % sequence identity threshold. For bacterial diversity analysis, taxonomic annotations utilized the RDP database v18 (Wang et al., 2007) based on representative OTU sequences. For fungal diversity, annotations used the UNITE database v9.0 (Nilsson et al., 2018). After annotation, alpha-diversity indices (Chao1, observed species, Shannon, and Simpson) were computed from the OTU table using QIIME scripts. Additionally, beta-diversity was assessed using QIIME's dedicated scripts.

2.6. Flavor evaluation of stinky acid samples

The sensory profile, including aroma and taste of the stinky acid samples, was assessed using a portable Electronic Nose (PEN3, Win Muster Airsense Analytics Inc., Schwerin, Germany) and a commercial Electronic Tongue (Taste-Sensing System SA 402B, Intelligent Sensor Technology Co. Ltd., Atsugi-shi, Kanagawa, Japan), as described by Cai et al. (2020) and Liu et al. (2023).

The E-nose's taste sensory indicators included six basic tastes: acidity, bitterness, astringency, saltiness, umami, and sweetness, along with three aftertastes: A (astringency), B (bitterness), and richness (umami), as identified by Wang et al. (2021).

Additionally, all ten E-nose sensors were utilized: W1C (aromatic organic compounds), W1S (methyl), W1W (sulfides), W2S (alcohols, aldehydes, and ketones), W2W (aromatic and sulfur compounds), W3C (ammonia and aromatics), W3S (long-chain alkanes), W5C (short-chain alkane aromatic components), W5S (broad sensitivity to nitrogen oxides), and W6S (mainly hydrides), as reported by Liu et al. (2023).

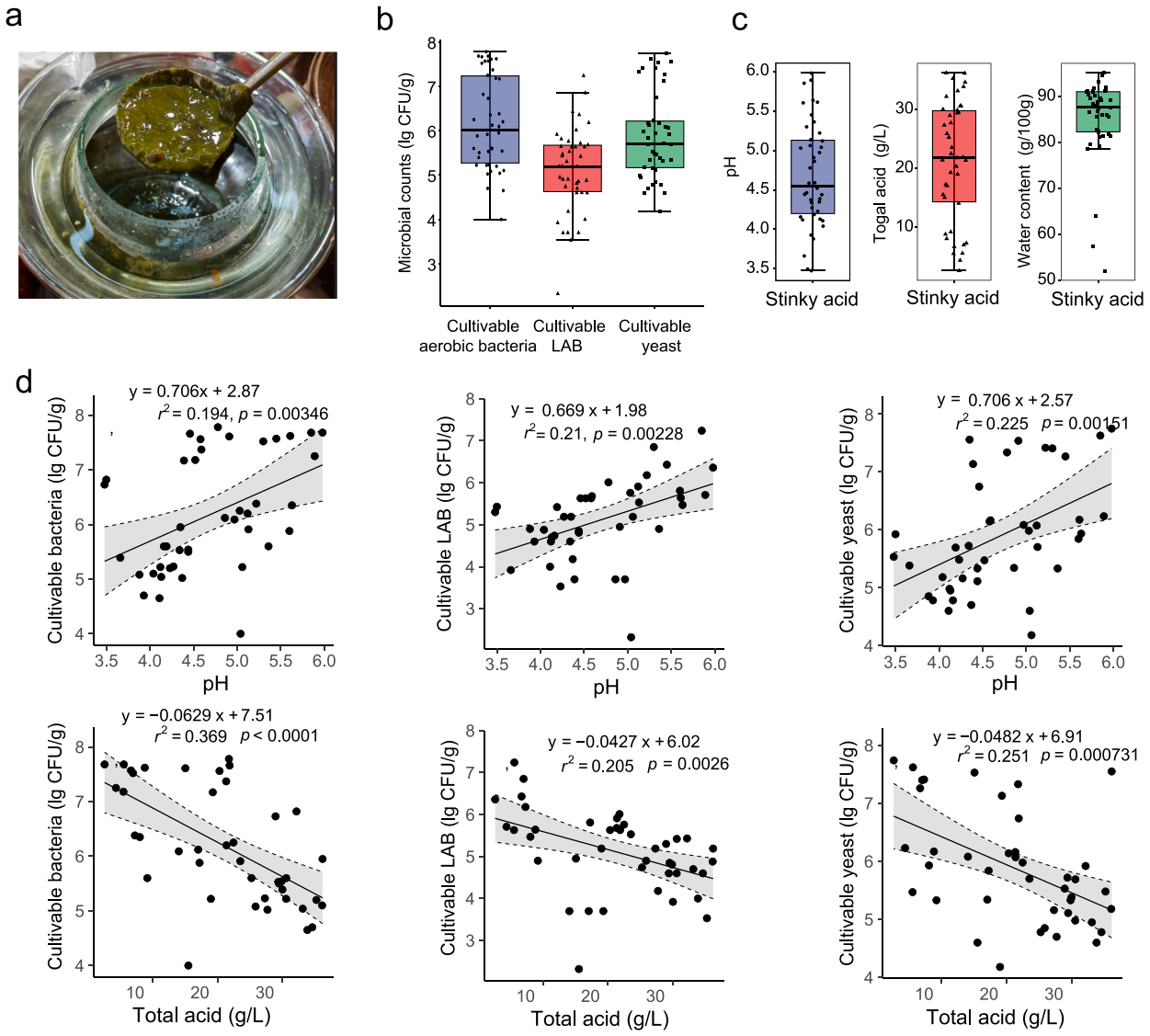

Fig. 1. Images showing the appearance of the collected stinky acid samples (a), culture-based microbial counts (b), physicochemical properties (c), and the linear regression relationship between the physicochemical properties and microbial counts (d). R^2 and p values of the linear regression analyses are shown. The black solid line indicates a significant linear relationship, with $p < 0.05$; gray areas represent the 95 % confidence intervals. Each black dot indicates a stinky acid sample. *: indicates $p < 0.05$, **: indicates $p < 0.01$, and ***: indicates $p < 0.001$.

2.7. Statistical analysis

Statistical analyses were conducted in RStudio v4.2.1. Linear regression analysis utilized ggtrendline v4.2.3 (https://github.com/PhDMeiwp/ggtrendline) in R. Mantel tests, using the linkET package in R (Huang et al., 2021), evaluated correlations between microbial profiles and sensory indices. Pairwise correlations between microbial genera and sensory indices were assessed with the psych package v2.4.1 (https://cran.r-project.org/web/packages/psych/index.html) in R. Cluster analysis, employing the k-means method, was conducted with factoextra (https://github.com/kassambara/factoextra) in R. Permutational multivariate analysis of variance (PERMANOVA; Adonis, permutation = 999) was carried out using pairwiseAdonis v0.4 in R (Irnawati et al., 2020). Additionally, the Wilcoxon test (Mann-Whitney) determined the statistical significance of differences in dominant taxa (Divine et al., 2013). Graphical representations were created using ggplot2 and ggpubr v0.6.0 in R (Kassambara et al., 2020).

3. Results

3.1. Physicochemical properties and culture-based microbial counts

Forty-two samples were collected, each exhibiting a green color, sticky texture (Fig. 1a), and distinct stinky smell. To assess microbial richness, microbial counts were conducted (Fig. 1b). Counts of total aerobic bacteria (log cfu/ml) ranged from 4 to 7.78, with LAB and yeasts ranging from 2.32 to 7.24 and 4.18 to 7.74, respectively.

Subsequently, pH, total acid content, and water content were measured (Fig. 1c). pH levels spanned from approximately 3.48 to 5.98, and total acid content varied from 2.63 to 36.19 g/L. Water content ranged from 52.08 to 95.17 g/100 g in the samples. These findings highlighted significant variability in physicochemical parameters and culture-based microbial richness among the samples.

Linear regression analysis provided deeper insights into the interplay between physicochemical properties and microbial richness (Fig. 1d). Results revealed a significant pattern: as pH increased, counts of total aerobic bacteria, LAB, and yeasts also increased ($p < 0.01$). Conversely, counts of total aerobic bacteria, LAB, and yeasts decreased as total acid content increased ($p < 0.01$). It's important to note pH represents the negative logarithm of hydrogen ion (H^+) concentration. This observation highlights contrasting trends in the relationship between pH, total acid, and microbial counts.

3.2. Microbial diversity and components

3.2.1. Bacterial diversity and community structure

Amplicon sequencing of a partial 16S rRNA gene was conducted to explore the bacterial diversity in stinky acid samples. High-throughput sequencing of the 42 stinky acid samples yielded a total of 1,539,932 high-quality sequences, with an average of 38,276 sequences per sample (Fig. S1). These sequences were classified into 32,565 OTUs, averaging 2,989 OTUs per sample (Fig. S1). These OTUs received annotations based on the latest RDP database (Fig. S1). These OTUs were annotated based on the latest RDP database (Fig. S1). In total, the 32,565 OTUs were annotated across 14 phyla, 26 classes, and 240 genera.

Subsequently, we calculated α-diversity indices, as presented in Fig. S1, based on a sequence depth of 21,010 sequences. To evaluate the adequacy of the sequencing depth, we analyzed rarefaction curves for

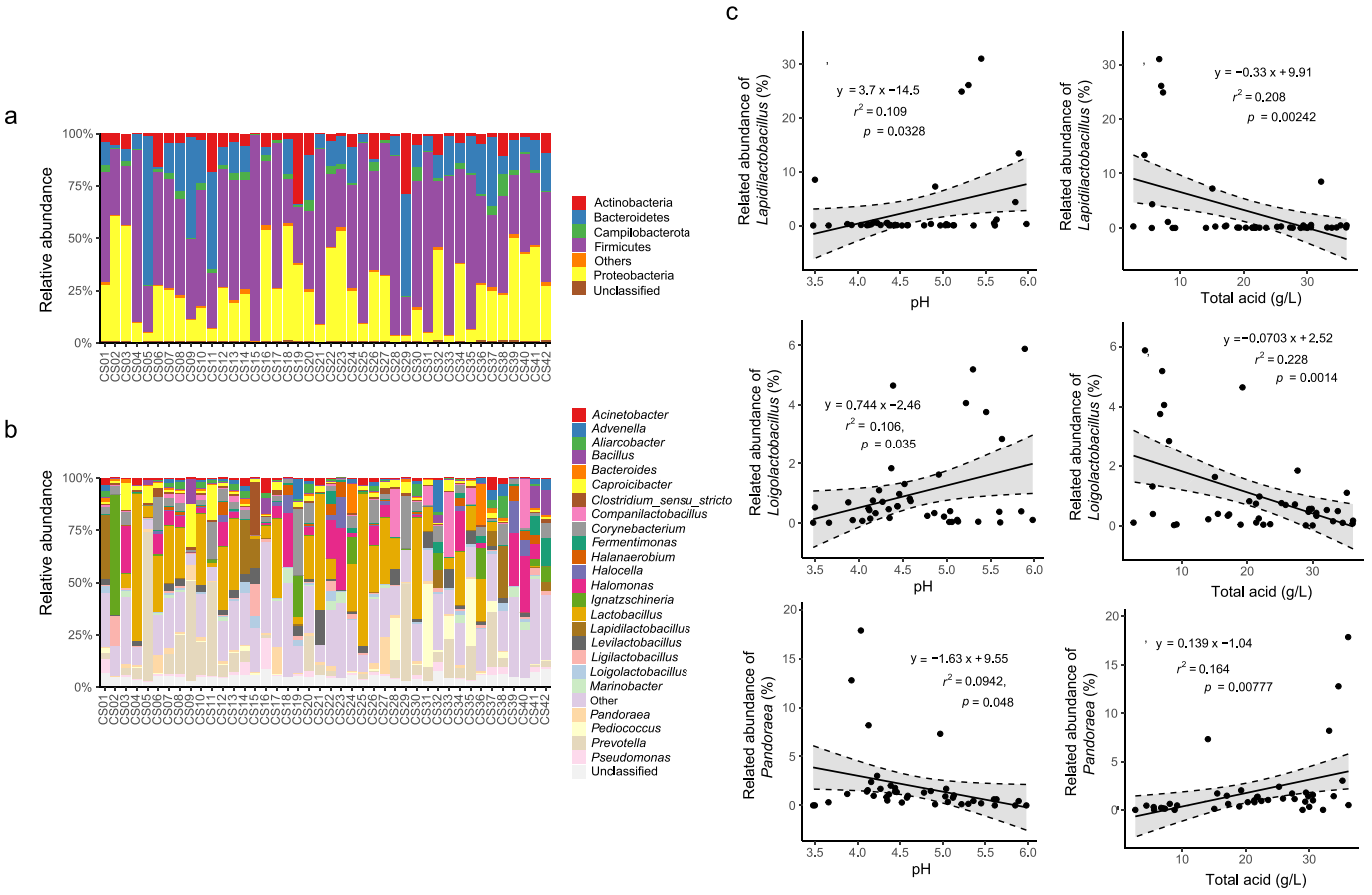

Fig. 2. Dominant bacterial phyla (a) and genera (b), and the relationships between the pH and total acid levels and the dominant bacterial genera (c). Fig. 2c: R^2 and p values of the linear regression analyses are shown; each black dot indicates a stinky acid sample. The black solid line indicates a significant linear relationship, with $p < 0.05$; gray areas represent the 95 % confidence intervals. Fig. 3a and 3b: Taxa with relative abundances < 1 % are summarized as "other".

the Shannon index (Fig. S2a) and the observed species index (Fig. S2b). The results confirmed that a sequencing depth of 21,010 was sufficient to capture the bacterial diversity in the 42 stinky acid samples.

Next, relative abundance of the phyla and genera was extracted to evaluate the microbial composition of stinky acid samples. In this study, taxa having relative abundances exceeding 1 % were deemed dominant. At the phylum level, five bacterial phyla showed average relative abundances exceeding 1 % (Fig. 2a), including Firmicutes (49.75 %), Proteobacteria (25.68 %), Bacteroidetes (15.29 %), Actinobacteria (5.95 %), and Campylobacterota (1.93 %). Among these, Firmicutes was identified as the most predominant.

At the genus level, twenty-four genera were found with average relative abundances surpassing 1 % (Fig. 2b), including *Lactobacillus* (16.57 %), *Prevotella* (10.08 %), *Halomonas* (5.99 %), *Corynebacterium* (5.30 %), *Caproicibacter* (3.05 %), *Ignatzschineria* (3.04 %), *Pediococcus* (3.00 %), *Lapidilactobacillus* (2.89 %), *Companilactobacillus* (2.72 %), *Levilactobacillus* (2.47 %), *Pandoraea* (1.91 %), *Advenella* (1.75 %), *Halocella* (1.56 %), *Pseudomonas* (1.48 %), *Aliarcobacter* (1.42 %), *Ligilactobacillus* (1.37 %), *Bacteroides* (1.36 %), *Clostridium_sensu_stricto* (1.24 %), *Bacillus* (1.18 %), *Marinobacter* (1.14 %), *Halanaerobium* (1.13 %), *Fermentimonas* (1.09 %), *Acinetobacter* (1.04 %), and *Loigolactobacillus* (1.03 %) (Fig. 2b). Notably, genera that potentially belonged to the LAB group accounted for an approximate cumulative relative abundance of 30.04 %.

Additionally, ten genera consistently occurred across all samples. These genera were identified as the core dominant genera, including: *Lactobacillus*, *Prevotella*, *Corynebacterium*, *Caproicibacter*, *Levilactobacillus*, *Pseudomonas*, *Ligilactobacillus*, *Bacteroides*, *Clostridium_sensu_stricto*, and *Loigolactobacillus*.

An analysis was conducted to delineate the relationship between dominant bacterial genera and the pH and total acid content of stinky acid (Fig. 2c). Through linear regression analysis, distinct trends were identified: an increase in the relative abundance of *Lapidilactobacillus* and *Loigolactobacillus* was observed with a higher stinky acid pH ($p < 0.05$). Conversely, a decrease in the relative abundance of *Pandoraea* was observed as the pH increased ($p < 0.05$). Furthermore, with an increase in the total acid content of stinky acid, a decrease in the relative abundance of *Lapidilactobacillus* and *Loigolactobacillus* was observed, whereas an increase in that of *Pandoraea* was observed ($p < 0.05$).

Through linear regression analysis, we recognized distinct trends: the relative abundance of *Lapidilactobacillus* and *Loigolactobacillus* increased with increasing stinky acid pH ($p < 0.05$). Conversely, the relative abundance of *Pandoraea* decreased as the pH increased ($p < 0.05$). Furthermore, the relative abundance of *Lapidilactobacillus* and *Loigolactobacillus* decreased with an increase in the total acid content of stinky acid, whereas that of *Pandoraea* increased ($p < 0.05$).

3.2.2. Fungal diversity and community structure

Based on high-throughput sequencing of the fungal ITS region, a total of 2,082,275 high-quality sequences was obtained, with an average of 49,578 sequences per sample (Fig. S1). These sequences were classified into 2,606 OTUs using a 97 % similarity threshold (Fig. S1). Annotation analysis identified the presence of 4 fungal phyla, 12 fungal classes, and 38 fungal genera across the stinky acid samples (Fig. S1). Although there were fewer fungal than bacterial taxa, the difference was not statistically significant (Fig. S1).

Furthermore, all four α-diversity indices of the fungal community were found to be lower than those of the bacterial α-diversity indices (Fig. S1). The Shannon and observed species index curves, shown in Fig. S3a and S3b, tended to plateau, suggesting that the selected sequencing depth was appropriate for capturing the fungal diversity present in the stinky acid samples.

The fungal phyla whose relative abundances exceeded 1 % were Ascomycota (94.58 %), Basidiomycota (3.01 %), and Mucoromycota (2.18 %, Fig. 3a). At the genus level, seventeen genera displayed relative abundances exceeding 1 %: *Pichia* (16.42 %), *Debaryomyces* (15.12 %), *Dipodascus* (11.64 %), *Saccharomyces* (9.47 %), *Yarrowia* (6.94 %), *Saccharomycopsis* (5.14 %), *Candida* (4.17 %), *Dekkera* (3.80 %),

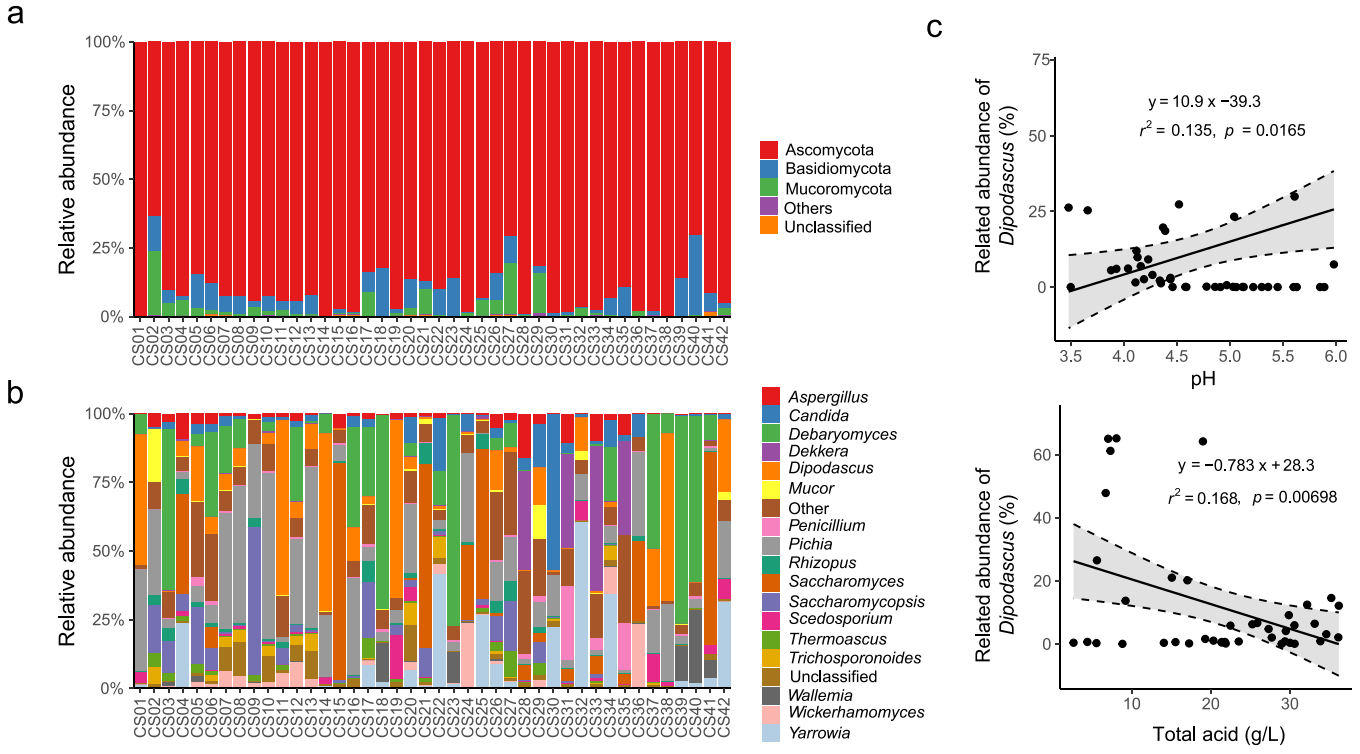

Fig. 3. Dominant fungal phyla (a) and genera (b), and relationships between pH and the dominant fungal genera (c). Fig. 3c: R^2 and p values of the linear regression analyses are shown; each black dot indicates a stinky acid sample. The black solid line indicates a significant linear relationship, with $p < 0.05$; gray areas represent the 95 % confidence intervals. Fig. 3a and 3b: Taxa with relative abundances < 1 % are summarized as "other".

Wickerhamomyces (2.56 %), *Aspergillus* (2.53 %), *Wallemia* (1.87 %), *Scedosporium* (1.61 %), *Penicillium* (1.60 %), *Rhizopus* (1.53 %), *Trichosporonoides* (1.36 %), *Thermoascus* (1.20 %), and *Mucor* (1.09 %, Fig. 3b). Among these genera, three core genera consistently occurred across all samples, namely *Pichia*, *Dipodascus*, and *Thermoascus*. Analysis of the dominant genera revealed significant variability in the fungal community structure of the stinky acid samples.

Regression analysis revealed clear trends: an increase in the relative abundance of *Dipodascus* was observed with a higher pH of the stinky acid, while it decreased with an increase in total acid content (Fig. 3c).

3.3. LAB isolates from stinky acid

To assess the LAB diversity within stinky acid, culture-based techniques were employed to isolate LAB strains, which were then identified through 16S rRNA sequencing analysis. Seventy-three strains were successfully isolated. Of these strains, only three exhibited a coccus morphology, whereas the rest were rod-shaped (Table S1). A hybrid approach involving blastn and phylogenetic tree analysis was employed to achieve a more detailed classification of these strains (Fig. 4).

Seventy-three strains identified in the study were classified into eight bacterial genera, including *Loigolactobacillus*, *Companilactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Ligilactobacillus*, and *Pediococcus*. Notably, *Loigolactobacillus* was the predominant genus, with 34 strains of *Loigolactobacillus coryniformis* identified, constituting 46.57 %. Included within the genus *Levilactobacillus* were five species: *Levilactobacillus angrenensis* (one strain, constituting 1.28 %), *Levilactobacillus brevis* (three strains, constituting 2.56 %), *Levilactobacillus tujiorum* (three strains, constituting 4.11 %), *Levilactobacillus yiduensis* (one strain, constituting 1.28 %), and *Levilactobacillus* sp. (two strains, constituting 8.22 %). Furthermore, within the genus *Ligilactobacillus*, one species, *Ligilactobacillus acidipiscis*, was identified, comprising eight strains and constituting 10.96 %. Within the genus *Companilactobacillus*, three species were identified, namely *Companilactobacillus alimentarius* (five strains, constituting 6.41 %), *Companilactobacillus nuruki* (one strain, constituting 1.28 %), and *Companilactobacillus baiquanensis* (one strain, constituting 1.28 %). The remaining genera each harbored fewer than five strains within their respective classifications.

Although isolates of two prominent LAB genera, *Lactobacillus* and *Lapidilactobacillus*, were identified through high-throughput sequencing, they were not detected via the culture-based approach. Additionally, the culture-based method effectively detected three genera: *Lacticaseibacillus*, *Lactiplantibacillus*, and *Lentilactobacillus*, each with a relative abundance of less than 1 % in high-throughput sequencing.

3.4. Flavor evaluation

3.4.1. Taste evaluation

An electronic tongue (E-tongue) system was utilized to evaluate the taste profile of the stinky acid samples (Fig. 5a). The sensor response values for bitterness, astringency, umami, aftertaste A, aftertaste B, and richness showed relatively minor variations, indicating a consistent pattern. In contrast, response values associated with sourness and saltiness demonstrated consistently greater variability. A widespread distribution of response values from the acidity sensor indicated a highly variable perception of sourness. This variability could likely be attributed to fluctuations in the microbial community structure during the natural fermentation process.

Pearson's correlation analysis was used to explore the relationships between E-tongue sensor response values and the dominant bacterial and fungal genera. The overall dominant bacterial communities showed substantial correlations with sensory attributes, including sourness, astringency, umami, saltiness, and the aftertaste of umami ($p < 0.05$), as confirmed through the Mantel test. Furthermore, significant correlations were observed between the dominant fungal communities and sensory attributes like sourness, saltiness, and umami ($p < 0.05$, Fig. 5b).

Notably, the findings revealed specific associations. The undesired flavor dimension of bitterness showed a positive correlation ($p < 0.05$) with the bacterial genus *Companilactobacillus* and the fungal genera *Thermoascus* and *Rhizopus*. Similarly, aftertaste B, linked to bitterness, demonstrated a positive correlation with *Companilactobacillus* and *Rhizopus* ($p < 0.05$, Figs. 5c and 5d). Another unfavorable flavor component, astringency, exhibited a positive correlation ($p < 0.05$) with the bacterial genera *Halomonas* and *Marinobacter*, and the fungal genus *Aspergillus*. Furthermore, saltiness showed a negative correlation ($p < 0.05$) with the LAB genera *Loigolactobacillus* and *Levilactobacillus*. Of particular significance, the characteristic sour taste demonstrated a positive correlation ($p < 0.05$) with *Debaryomyces* and a negative correlation with *Dipodascus*.

3.4.2. Odor evaluation

Each stinky acid sample underwent E-nose simulated sensory analysis (Fig. 6a). As shown in Fig. 6a, most sensor response values were minimal. Conversely, sensors W1W and WS (W1S, W2S, W5S) showed notably higher response values, indicating elevated concentrations of aromatic constituents such as sulfides, methyl compounds, alcohols, aldehydes, ketones, and nitrogen oxides in the samples. Among these, sulfides, potentially responsible for the characteristic odor profile, emerged as the most prominent contributor detected by W1W. Dominant bacterial communities showed a highly significant correlation with WS (W5S, W2S) response values ($p < 0.01$) and significant correlations with WC (W1C, W3C, W5C), W1W, and W6S ($p < 0.05$). Fungal communities showed significant correlations with W1S and W2S sensor response values ($p < 0.05$) (Fig. 6b).

Subsequently, focus was on individual dominant genera. *Pediococcus*, along with *Rhizopus* and *Thermoascus*, showed a significant negative correlation with WS (W1S, W2S, W5S) sensor response values ($p < 0.05$, Fig. 6c and 6d). *Ignatzschineria* and *Ligilactobacillus* demonstrated a positive, significant correlation with the W5S sensor response value ($p < 0.05$). Notably, only *Rhizopus* and *Thermoascus* had significant correlations with the W1W sensor response values ($p < 0.05$). In contrast, no dominant bacterial genera showed significant correlations with the W1W sensor response value ($p > 0.05$).

3.5. Differences between types of stinky acid

The E-sensory profile of stinky acid showed significant variability in response values for attributes including sourness, saltiness, W1W response, and W5S response, among others. Using the k-means method based on Bray distances of E-sensory indices, the 42 stinky acid samples were successfully clustered into three distinct types (Fig. 7a and 7b). Bray–Curtis distances confirmed highly significant differences among the three types of stinky acid samples ($p < 0.001$). Random forest analysis and the Wilcoxon test were employed to determine the characteristics of the three types of stinky acid (Fig. 7c, 7d, and 7e). Type 1 stinky acid showed the highest response values for W5S, W1W, W1S, and W2S sensors, and the lowest for WC sensors (W1C, W3C, and W5C) ($p < 0.01$). Conversely, type 3 showed the highest response values for WC sensors (W1C, W3C, and W5C). Type 2 exhibited the highest relative abundance of *Pandoraea* and *Wickerhamomyces* ($p < 0.01$).

ROC analysis and leave-one-out cross-validation demonstrated W1W's sufficient discriminatory power for stinky acid samples. However, in the absence of sensory indices, a single microbial genus proved insufficient to differentiate among the three types of stinky acid (Fig. 7f, 7g, and 7h). *Pandoraea* and *Bacteroides* were identified as key markers, offering enhanced discriminatory power for pairwise differentiation among type 1, type 2, and type 3 stinky acids.

4. Discussion

The traditional fermented condiment stinky acid originates primarily from the southern region of Guizhou Province. Its unique microbial

Fig. 4. Neighbor-joining (NJ) phylogenetic tree of LAB strains from stinky acid and their closest type material based on 16S rRNA gene sequences. The NJ tree was constructed with MEGA X using 1000 bootstrap replicates. Alignment of the 16S rRNA gene sequences was performed using Mafft v7.475. The accession numbers of 16S rRNA sequences used to construct NJ tree were provided in square brackets.

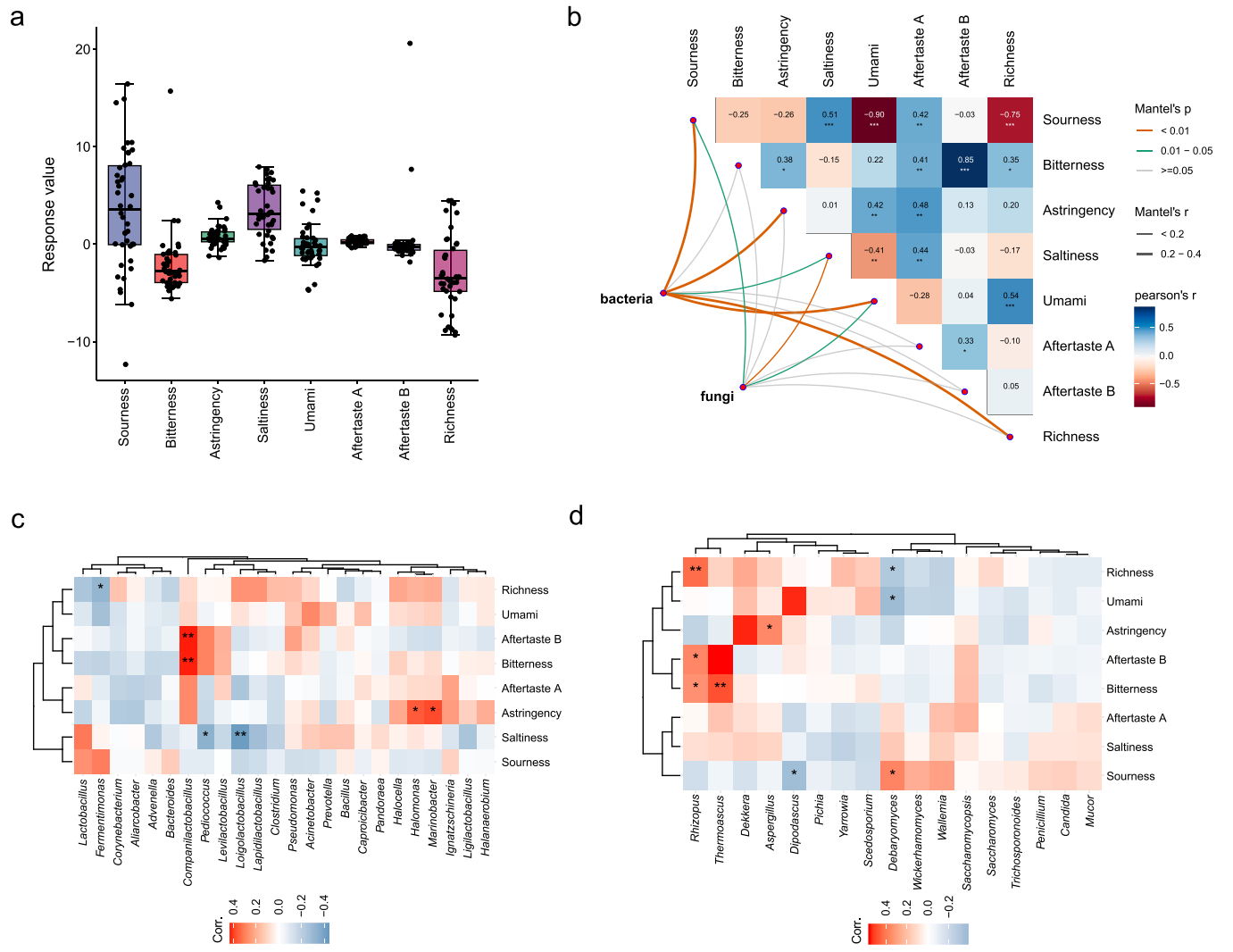

Fig. 5. Box plot for taste properties (a), and correlation analysis between E-tongue indices and the overall microbial groups (b), dominant bacterial genera (c), and dominant fungal genera (d). *: indicates $p < 0.05$; **: indicates $p < 0.01$, ***: indicates $p < 0.001$. Corr. = correlation coefficient.

diversity is likely shaped by the specific environmental conditions and raw materials of this area, distinguishing it from other fermented foods. To investigate the microbial profile and flavor properties of stinky acid comprehensively, a combination of high-throughput sequencing, culture-based methods, and bionic E-tongue and E-nose analyses was employed.

Numerous traditional fermented foods from specific regions possess unique odors, including grey sufu, Chouguiyu, and Yan-dong-gua (fermented wax gourd). *Acinetobacter* and *Enterococcus*, consistently detected throughout grey sufu's fermentation process, are considered resident bacteria. Furthermore, the dominant bacterial genus *Pseudomonas* has been linked to the characteristic stinky aroma of grey sufu (Ding et al., 2023). Chouguiyu is characterized by the dominance of *Vibrio*, *Psychrobacter*, *Psychrilyobacter*, and *Fusobacterium*, with *Psychrilyobacter* notably influencing flavor formation (Yang et al., 2021). While grey sufu and Chouguiyu fermentation transform proteins into amino acids for a unique flavor, stinky acid distinguishes itself by using impatiens as a primary raw material. This study's high-throughput sequencing revealed 24 dominant bacterial and 14 dominant fungal genera in diverse stinky acid samples. A comparison with other fermented foods revealed commonalities and differences. Compared to grey sufu, Chouguiyu, and Yan-dong-gua, *Advenella*, *Aliarcobacter*, *Caproicibacter*, and *Halocella* are uniquely dominant only in stinky acid, highlighting its unique microbial community (Ding et al., 2023; Lan et al., 2009; Yang et al., 2021). Stinky acid and grey sufu share dominant genera including *Pseudomonas*, *Pediococcus*, *Bacillus*, and *Lactobacillus*, albeit with varying levels of abundance (Ding et al., 2023), whereas, stinky acid, Yan-dong-gua and Chouguiyu only exhibit a dominant abundance of *Lactobacillus* (Lan et al., 2009; Yang et al., 2021). The dominance of the LAB genus *Lactobacillus* in all four stinky fermented foods suggests a common role in enhancing sourness. In addition, the LAB group accounted for an approximate cumulative relative abundance of 30.04 %. LAB is also found in pickled vegetables, zha-chili, and sour soup (Guo et al., 2021; Cai et al., 2021; Zhang et al., 2023; Lin et al., 2022). This group of bacteria, characterized by unique fermentative behavior yielding lactic acid as the primary end product, influences both sour taste and aroma development. In this study, correlation analysis was used to speculate on the influence of certain lactic acid bacteria, including *Lactobacillus*, on the flavor of stinky acid.

Factors contributing to microbial diversity include pH, salt content, and temperature (Shu et al., 2022). Our study measured pH and total acid across 42 samples, prompted by stinky acid's characteristic sour taste. Simultaneously, culture-based microbial counts were conducted, including total aerobic bacteria, LAB, and yeast. Regression analysis showed a positive correlation between pH and microbial richness ($p < 0.05$), and a negative correlation between total acid content and microbial richness. This highlighted the role of acidity as a potential constraint on microbial counts. Interestingly, regression analysis

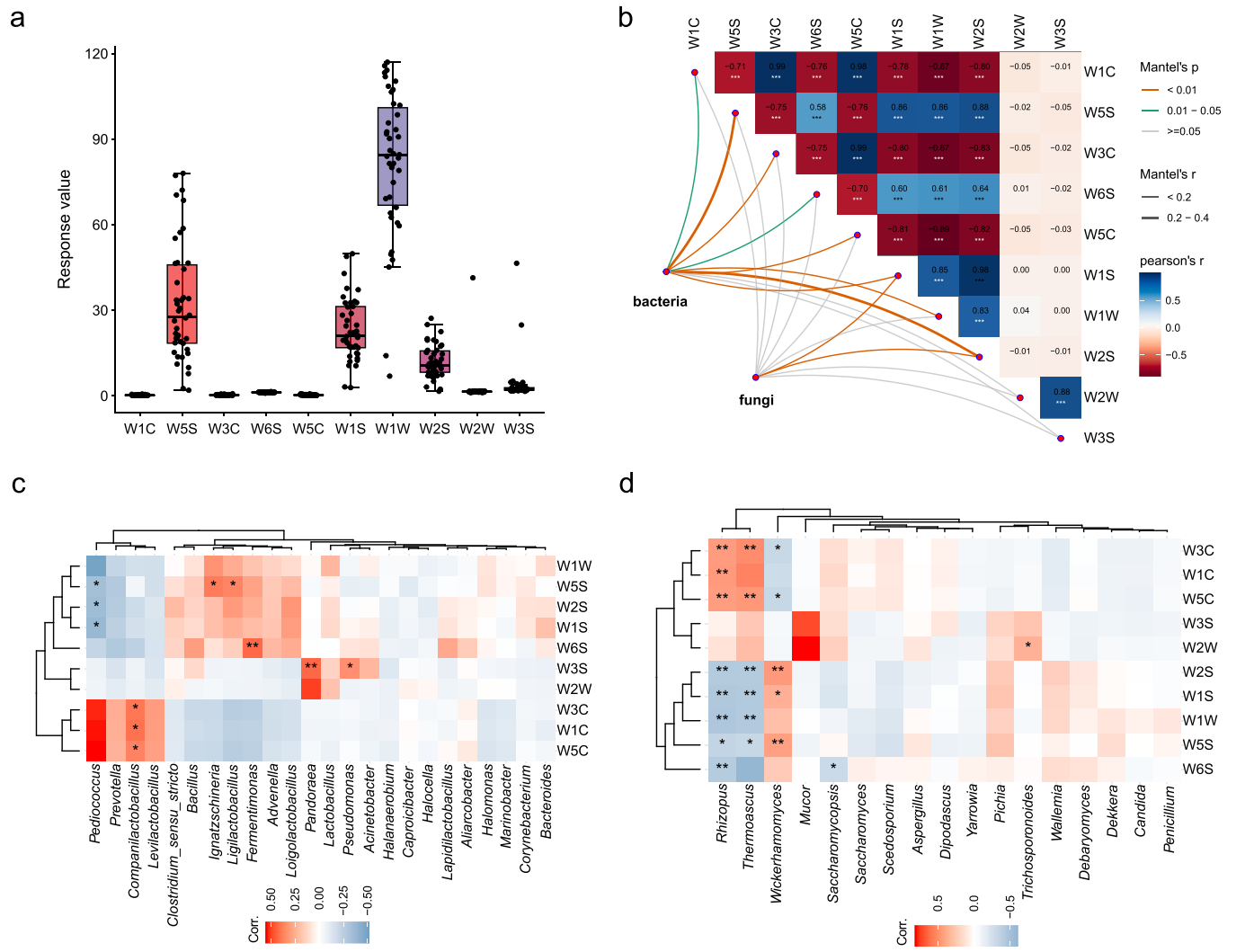

Fig. 6. Box plot for odor properties (a), and correlation analysis between E-nose indices and the overall microbial groups (b), dominant bacterial genera (c), and dominant fungal genera (d). *: indicates $p < 0.05$; **: indicates $p < 0.01$, ***: indicates $p < 0.001$. Corr. = correlation coefficient.

identified significant correlations between acidity and specific taxa: increased acidity was associated with decreased relative abundance of *Lapidilactobacillus*, *Loigolactobacillus*, and *Dipodascus* ($p < 0.05$). Notably, the dominant bacterial genus *Pandoraea* showed a positive correlation with acidity ($p < 0.05$), indicating notable resistance to high acidity. This is significant as *Pandoraea* spp. typically thrive in liquid mediums at pH levels as low as 4. This suggests *Pandoraea* spp.'s potential adaptation in stinky acid, highlighting their resilience to low pH (Peeters et al., 2019). Through LAB isolation and identification, stinky acid-dominant LAB *Loigolactobacillus* was identified as *Loigolactobacillus coryniformis*, listed in the 2019 QPS update, underscoring its potential food production applications.

Although stinky acid is known for its distinctive smell, it notably offers an appealing taste as a condiment. Despite sourness being a characteristic flavor of stinky acid, considerable variability in sourness exists among samples produced through natural fermentation. This study's correlation analysis revealed important links between specific microbes and stinky acid's sensory attributes. Specifically, the dominant yeast *Debaryomyces* was positively correlated with sourness, aligning with findings that *Debaryomyces* spp. contribute to acid production (Santos de Almeida et al., 2023). This is likely due to their ability to convert sugars into organic acids via glycolysis, the TCA cycle, and the pentose phosphate pathway. Additionally, *Debaryomyces* spp. have been shown to enhance the flavor of cheese and fermented sausages (Gori et al., 2012; Santos de Almeida et al., 2023). Consequently, *Debaryomyces* spp. could be utilized to enhance stinky acid's sour taste. Interestingly, the dominant LAB *Loigolactobacillus* showed a negative correlation with saltiness, suggesting its utility in reducing stinky acid's salty taste.

Defective flavors negatively impact food quality. Correlation analysis demonstrated that *Companilactobacillus*, *Thermoascus*, and *Rhizopus* were positively associated with bitterness and its aftertaste. Furthermore, *Halomonas*, *Marinobacter*, and *Aspergillus* were positively correlated with the undesirable taste of astringency. Therefore, these microorganisms may contribute to the development of unfavorable tastes in stinky acid. Despite the identification of numerous taxa potentially promoting off-flavors, it is noteworthy that, minimal variations were observed in terms of bitterness, aftertaste B, and astringency across different samples. Remarkably, local consumers scarcely perceive the bitterness and astringency of stinky acid, suggesting that these off-flavors may not be the primary determinants affecting product quality.

Stinky acid's prominent characteristic is its distinct odor, largely influenced by microbial fermentation and various metabolic pathways. Research suggests sulfides are the characteristic volatile compounds responsible for the distinctive odors. Liu et al. illustrated that fermented stinky tofu contains sulfide variants and indole, contributing to its unfavorable aroma (Liu et al., 2012). Additionally, dimethyl disulfide and methyl thiopropionaldehyde were detected in Yan-gui-yu (Wu et al.,

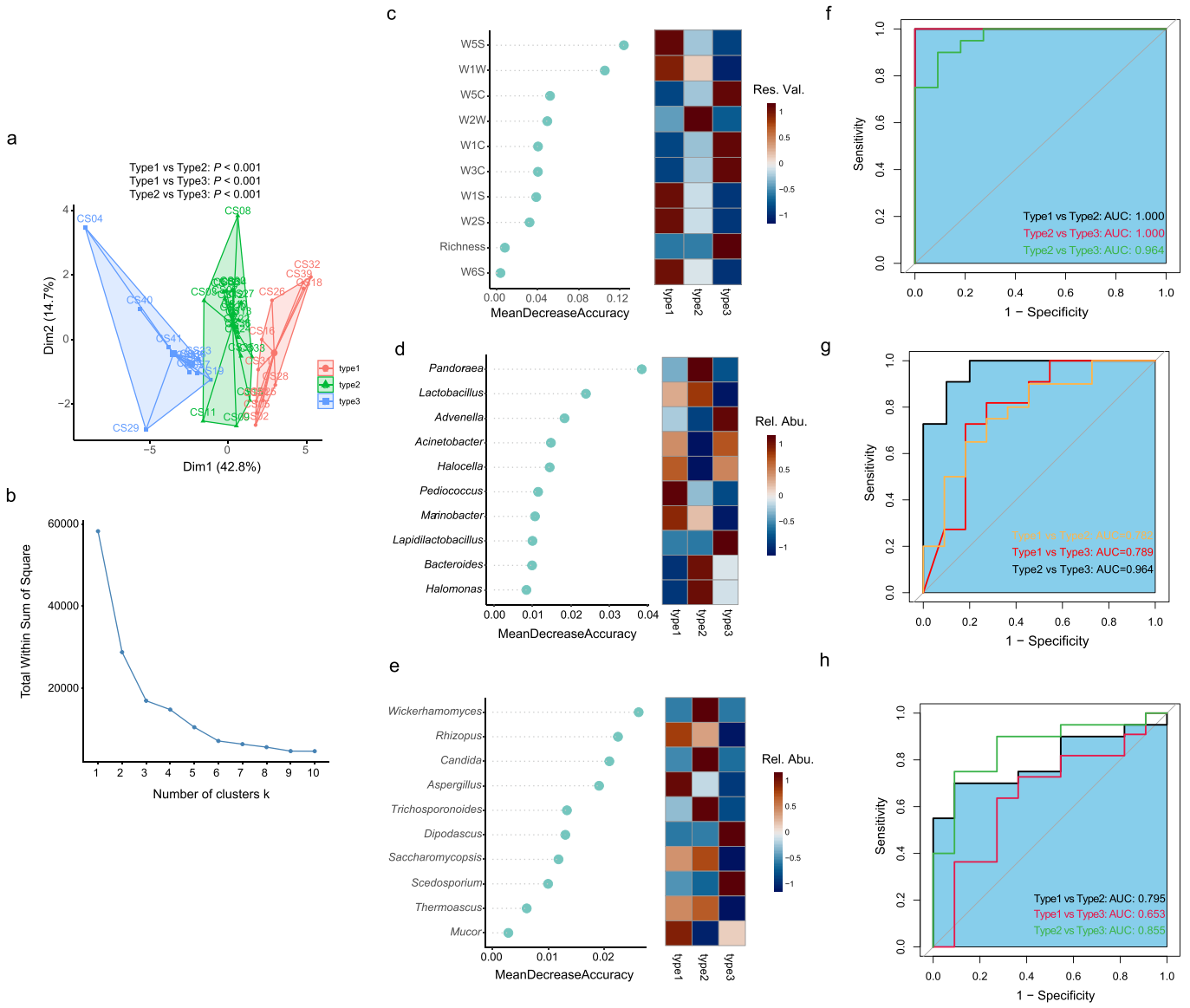

Fig. 7. Cluster analysis of stinky acid, and biomarkers for the pairwise discrimination of the three types of stinky acid. a, b Cluster analysis of stinky acid based on E-sensory indices using the k-means method. c Classification performance of the top 10 discriminant E-sensory indices using a random forest model and heatmap based on the response values of these indices. d Classification performance of the 10 most discriminant bacterial genera using a random forest model and heatmap based on the relative abundance of the bacterial genera. e Classification performance of the 10 most discriminant fungal genera using a random forest model and heatmap based on the relative abundance of these fungal genera. f Receiver operating characteristic (ROC) curve analysis for the E-nose marker W5S identified based on random forest analysis. g ROC analysis for the bacterial genus marker *Pandoraea* identified based on random forest analysis. h ROC analysis for the fungal genus marker *Wickerhamomyces* identified based on random forest analysis. Rel. Abu. = Relative abundance; Res. Val. = Response values.

2019; Zhou et al., 2019). Sulfide compounds, with low odor thresholds and strong flavor profiles, are key contributors to the aromas of products like cheeses, beer, and baijiu. Our study used E-nose sensors to assess stinky acid's flavor characteristics, showing the highest response for W1W, indicative of sulfides, confirming their role as distinctive volatile flavor constituents in stinky acid. Interestingly, correlation analysis showed a negative association between the fungal genera *Rhizopus* and *Thermoascus* and the W1W response value ($p < 0.001$), suggesting these fungi may reduce sulfide content, thus diminishing stinky acid's odor. Furthermore, *Pediococcus*, *Rhizopus*, and *Thermoascus* were found to enhance aromatic constituents detectable by WC sensors. Additionally, *Companilactobacillus* bacteria were associated with enhancing aromatic constituents. Reclassified from *Lactobacillus* in 2021, *Companilactobacillus* has been detected in fermented foods like zha-chili (Song et al., 2021) and pickles (Shang et al., 2022), and the human gut (Zhang et al., 2021). In this study, *Companilactobacillus* primarily consisted of *Companilactobacillus alimentarius*. Therefore, beyond influencing off-flavors, *Companilactobacillus*, *Rhizopus*, and *Thermoascus* could enhance stinky acid's aroma and reduce its characteristic odor, improving palatability for more consumers.

Microorganisms play a pivotal role in influencing the flavor of fermented foods. This study observed considerable variability in stinky acid's microbial communities, including both bacterial and fungal. The variability can be attributed to using unsterilized raw materials, pottery jars, and the absence of starter cultures. Consequently, significant differences in the E-sensory indices of stinky acid samples were observed. Using the E-sensory indices, the collected stinky acid samples were classified into three distinct types through the k-means method. Sensory indicators like W1W, capable of detecting sulfides and nitrogen oxides, effectively discriminated these three stinky acid types. Although individual microbial genera may lack sufficient discriminatory capability, combining *Pandoraea* and *Bacteroides* improves discriminatory power

for differentiating among stinky acid types 1, 2, and 3.

5. Conclusion

This study delivers a thorough examination of the microbial diversity and flavor characteristics of stinky acid, a unique fermented condiment from Guizhou Province, shedding light on the complex interplay between these aspects. The research demonstrates that the microbial loads of lactic acid bacteria (LAB), total aerobic bacteria, and yeast exhibit negative correlations with the acidity in stinky acid. Despite considerable variability in the microbial communities of stinky acid, ten core dominant bacterial genera, including *Lactobacillus*, *Prevotella*, *Corynebacterium*, among others, and core fungal genera such as *Pichia*, *Dipodascus*, and *Thermoascus*, were identified. The research further establishes a link between these dominant microbes and specific flavor attributes, identifying sulfides as critical volatile compounds shaping the unique odor of stinky acid. Notably, the fungi *Rhizopus* and *Thermoascus* were implicated in moderating this distinct aroma, while also, along with *Companilactobacillus*, augmenting aromatic qualities. Additionally, *Debaryomyces* spp. emerged as a significant contributor to the sourness profile, underscoring its role in flavor modulation. This investigation not only deepens the understanding of stinky acid's fermentation ecology but also opens avenues for refining its sensory appeal through microbial management.

Data Statement

The high-throughput sequencing data can be downloaded from the NCBI SRA database under accession number SRR26079047-SRR26079088. The 16S rRNA gene sequences for the LAB strain can be obtained from NCBI GenBank using the accession number OR563707-OR563779.

CRediT authorship contribution statement

Zhendong Zhang: Writing – review & editing, Writing – original draft, Visualization, Funding acquisition. **Huijun Zhao:** Supervision, Investigation. **Renzhi Zhu:** Software, Resources, Methodology. **Shaojing Cheng:** Supervision, Resources, Methodology. **Yuanqi Yu:** Validation, Supervision, Resources. **Lan Xiang:** Formal analysis, Data curation. **Zhipan Xiang:** Software, Formal analysis. **Zhuang Guo:** Visualization, Funding acquisition, Data curation. **Yurong Wang:** Writing – review & editing, Visualization, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101311.

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