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Original Research Article

Mixed-Culture Fermentation Enhances the Flavor Profile and Quality of Highland Barley Whiskey: Insights from Comprehensive Two-Dimensional Gas Chromatography–Time-of-Flight Mass Spectrometry (GC×GC–TOFMS)

Hui Zhu[1], Pei Meng[1], Jie Li[1], Panling Liao[1], Huan Fang[1], Ting Liang[1], Rongfei Wang[1], Weiming Jiang[2], Huan Xiao[3], Ning Wang[1,4*]

[1]Sichuan Province Engineering Technology Research Center of Liquor-Making Grains, School of Food and Liquor Engineering, Sichuan University of Science and Engineering, Yibin 644000, China
[2]Yingxiu People's Tea Industry Co., Ltd. Wenchuan 623003, China
[3]Xifeng Agricultural Science & Technology Development Co., Ltd., Cuiping District, Yibin, Sichuan, China
[4]Yibin Sichuan University of Science and Engineering Industrial Technology Research Institute, Yibin 644000, China

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Abstract: This study investigated the effects of single-strain and mixed-culture fermentation on the flavor profile and quality of highland barley whiskey. A yeast strain (LC3) was isolated from pit mud of traditional Chinese Baijiu, and compared with commercial whiskey yeast (W19) in both single and mixed fermentation systems. Highland barley and malt were mixed at a 1:1 ratio and fermented under controlled conditions. Flavor evaluation was conducted using a combination of electronic nose (E-nose) analysis, sensory panel assessment with flavor wheel development, and comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC×GC–TOFMS). The results showed that LC3 produced superior ethanol yield and enhanced ester formation compared to W19. Mixed-culture fermentation (LC3 + W19) significantly increased the concentration and diversity of key volatile compounds, including ethyl hexanoate and ethyl decanoate, contributing to enhanced fruity and floral notes while suppressing undesirable volatiles. E-nose PCA analysis revealed distinct odor profiles among the fermentation groups, supporting the synergistic effect of mixed cultures. This study demonstrates that combining pit mud–derived and commercial yeast strains provides a viable strategy to enhance flavor complexity, ester content, and overall sensory quality of highland barley whiskey. These findings offer a theoretical basis and practical guidance for optimizing whiskey fermentation and improving the quality of highland barley–based distilled spirits.

Keyword: Highland Barley Whiskey, Mixed-Culture Fermentation, Yeast Strain LC3, GC×GC–TOFMS; Sensory Analysis.

1. INTRODUCTION

Highland barley (Qingke), a cereal grain native to the Tibetan Plateau, has gained attention in whiskey production due to its high protein and fiber content, along with its unique nutritional profile(Yin & Zhang, 2024; W. J. Zhang, Li, & Zhao, 2024). Whiskey produced from highland barley often exhibits regional characteristics such as subtle grain aroma, light smokiness, and mild sweetness. However, its flavor complexity remains limited, presenting opportunities for quality enhancement(Liu, Feng, & Wu, 2020; Yin & Zhang, 2024). Previous studies suggest that blending Australian malt with lightly roasted highland barley can introduce delicate floral and fruity notes, thereby enriching the final aroma profile.

Yeast plays a pivotal role in alcoholic fermentation, not only converting sugars into ethanol and carbon dioxide but also generating a wide array of volatile flavor compounds—including esters, higher alcohols, aldehydes, and ketones—that define the spirit's aromatic structure and sensory identity(Huang, Zhang, &

***Corresponding Author:** Ning Wang
Sichuan Province Engineering Technology Research Center of Liquor-Making Grains, School of Food and Liquor Engineering, Sichuan University of Science and Engineering, Yibin 644000, China

Wang, 2025; Jafari, Moradi, & Hassan, 2024; Ma, Chen, & Huang, 2024). In both whiskey and traditional Chinese Baijiu production, the selection and combination of yeast strains are critical for shaping the final product's flavor(Li, Shi, Guang, Ge, & Yan, 2021; Y. Zhang & Liu, 2023). Chinese Baijiu is renowned for its complex and intense aroma, which arises from a diverse microbial community in fermentation pit mud. These naturally adapted microorganisms, particularly yeast strains, are key producers of ester compounds and other flavor-active metabolites.

This study isolated and characterized a flavor-producing yeast strain from Baijiu pit mud and applied it—both individually and in combination with commercial whiskey yeast—in highland barley whiskey fermentation. By integrating modern analytical techniques (E-nose, sensory analysis, and GC×GC–TOFMS), we systematically evaluated the flavor outcomes of different fermentation regimes. The research aims to clarify the potential of` mixed-culture fermentation in enhancing ester synthesis and overall flavor complexity, thereby offering theoretical insights and practical guidance for improving highland barley whiskey quality.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Sources included: pit mud from a strong-aroma-type Baijiu fermentation, commercial whiskey yeast strain W19, highland barley (Qingke, Qinghai origin), and Australian malt.

2.2. Experimental Procedures

2.2.1. Isolation and Screening of Yeast Strains

Pit mud samples were collected using a five-point sampling method to ensure representativeness(Smith & Brown, 2024; Wu, Li, & Chen, 2022). After serial dilution, suspensions were spread onto YPD agar plates and incubated at 30°C for 24 h. Isolated colonies were inoculated into YPD broth for further characterization.

2.2.1.1. Flavor-Based Screening

Each isolated yeast strain was used for single-strain fermentation in sterilized highland barley wort under static conditions at 28°C for 7 days. The resulting fermented samples were filtered, distilled, and evaluated by a trained sensory panel (5 members) using blind tasting. Attributes included aroma intensity, harmony, palate purity, and overall acceptability, scored on a 10-point scale.

2.2.1.2. Fermentation Capacity Screening

A tetrazolium chloride (TTC) overlay assay was used to qualitatively assess alcohol production capacity based on color development(Jones & Taylor, 2022; Nguyen & Patel, 2021). Strains showing rapid and intense red coloration were selected for quantitative fermentation tests in malt extract medium. Ethanol content was measured after 3 days of fermentation at 30°C with shaking (150 rpm).

2.2.1.3. Molecular Identification

Selected yeast strains were identified morphologically and via sequencing of the internal transcribed spacer (ITS) region. PCR amplification was performed using fungal-specific primers, and phylogenetic analysis was conducted using MEGA11.0 software with reference sequences from NCBI.

2.2.2. Highland Barley Whiskey Production

Based on optimized protocols, fermentation was carried out using three inoculum strategies:
Group A: Commercial yeast W19 (single strain)
Group B: Pit mud–derived yeast LC3 (single strain)
Group C: Mixed culture of W19 and LC3 (1:1 ratio)

Each group was prepared in triplicate. Highland barley and malt were mixed (1:1), mashed, and saccharified. After cooling, yeast was inoculated, and fermentation proceeded at 28°C for 10 days. The fermented mash was distilled under controlled conditions, and the heart cut (collected between 30 s and 8 min after distillation began) was retained as the final spirit.

2.2.3. Flavor Analysis

2.2.3.1. Electronic Nose (E-Nose) Analysis

A portable E-nose system with 10 metal oxide sensors was used. Samples (1 mL) were equilibrated in headspace vials, and sensor responses were recorded during the stable phase (57–59 s). Principal component analysis (PCA) was applied to differentiate aroma profiles among groups(Kim, Lee, & Park, 2019; Wang & Zhao, 2020).The performance characteristics of each sensor are shown in Table. 1.

Table 1: Performance characteristics of electronic nose sensor

Array Serial Number	Sensor Name	Representative Substance of Sensor	Sensor Performance Description
1	W1C	Aromatic	Sensitive to aromatic compounds
2	W5S	Broadrange	Responsive to nitrogen oxides
3	W3C	Aromatic	Exhibit sensitivity to ammonia compounds and aromatic hydrocarbons
4	W6S	Hydrogen	Selective response to hydrides
5	W5C	Arom-aliph	High sensitivity to short-chain alkanes and aromatic constituents

Array Serial Number	Sensor Name	Representative Substance of Sensor	Sensor Performance Description
6	W1S	Broad-methane	Pronounced reactivity toward methyl derivatives
7	W1W	Sulphur-organic	Strong affinity for sulfide compounds
8	W2S	Broad-alcohol	Differential responsiveness to alcohols, aldehydes, and ketones
9	W2W	Sulph-chlor	Specific interaction with aromatic components and organic sulfides
10	W3S	Methane-aliph	Preferential detection of long-chain alkanes

2.2.3.2. Flavor Wheel Development

A sensory panel of 12 trained assessors evaluated the spirits using descriptive analysis. Panelists generated terms for aroma, taste, mouthfeel, and aftertaste. Redundant or irrelevant terms were removed, and a flavor wheel was constructed by categorizing the remaining descriptors with reference to established wheels for whiskey and Baijiu(Chen & Xu, 2020; Lopez & Hernandez, 2021; R. Singh & Kumar, 2019).

2.2.3.3. GC×GC–TOFMS Analysis

GC×GC–TOF MS analysis was employed to separate and identify esters, alcohols, aldehydes, alkanes, and aromatic compounds, followed by heatmap visualization and statistical analysis(Martinez & Garcia, 2023; Thompson & Roberts, 2022; Zhao & Deng, 2019). Volatile compounds were analyzed using a GC×GC–TOFMS system equipped with a DB-5MS primary column (30 m × 0.25 mm, 0.25 μm) and a DB-17MS secondary column (1.5 m × 0.18 mm, 0.18 μm). Helium was used as carrier gas at 1 mL/min. The oven temperature program started at 35°C (hold 5 min), increased at 3°C/min to 230°C (hold 5 min). The modulator offset was 20°C with a 4 s cycle. Mass spectra were acquired in EI mode at 70 eV, scanning m/z 35–550.

3. RESULTS AND DISCUSSION

3.1. Yeast Screening and Identification

Eight yeast strains were isolated from pit mud. Four strains (LC1, LC2, LC3, and LC7) scored above 8.0 in the sensory evaluation(Park & Kim, 2020; H. Singh & Lee, 2021), showing pronounced ester aroma, harmonious profile, and clean palate (Table. 2). The selected candidate strains were subjected to qualitative analysis using the TTC method. By comparing the intensity of the color change, it was observed that LC3 and LC7 exhibited deeper coloration than LC1 and LC2 (Fig. 1A)Among these, LC3 exhibited the highest ethanol yield in fermentation tests (Fig. 1C). And LC3 was identified via ITS sequencing as *Kazachstania bulderi* (99.82% similarity) (Fig. 1D). The LC3 colonies were moist, smooth, and viscous, with well-defined edges, presenting an overall round, milky-white appearance. Under the microscope, the cells appeared as single, ovoid-shaped units (Fig. 1B). LC3 produced the highest alcohol content, confirming its ethanol-producing capacity as indicated by the TTC test(Adams & O'Neill, 2020; Roberts & Wilson, 2021); therefore, LC3 was selected as the strain for subsequent whiskey production.LC3 was selected for subsequent brewing experiments.

Table 2: Comprehensive scores of wines fermented by 8 yeast strains

Strains	Aroma intensity	Aroma harmony	Taste authenticity	Overall acceptability	Average score
LC1	8.5	8.2	8.4	8.6	**8.43**
LC2	8.6	8.4	8.5	8.7	**8.55**
LC3	8.5	8.4	8.5	8.6	**8.5**
LC4	7	6.9	7.2	7.1	7.05
LC5	7.2	7	7.1	7.3	7.15
LC6	6.8	7.1	6.9	7	6.95
LC7	8.4	8.3	8.3	8.5	**8.38**
LC8	7.1	7.2	7	6.9	7.05

3.2. Sugar Utilization during Fermentation

Mixed-culture fermentation (Group C) showed the fastest sugar consumption and the lowest final sugar content, indicating enhanced metabolic efficiency and synergy between W19 and LC3 (Fig. 2A).

3.3. E-Nose Analysis

As shown in the Electronic Nose PCA diagram (Fig. 2B), the contribution rate of the first principal component (PC1) is 80.7%, and that of the second principal component (PC2) is 18.7%, with a cumulative contribution rate reaching 99.4%. This indicates that the two principal components of PCA can largely reflect the overall profile of the samples, while the differences between samples are mainly represented by the distances along the horizontal axis. The diagram includes three groups, each containing three replicate highland barley wine samples, with each sample analyzed in triplicate. Group A consists of wine samples obtained from single-strain fermentation using LC3 , Group B from single-strain fermentation using commercial yeast W19, and Group C from mixed fermentation using both

commercial yeast W19 and LC3. The three groups are distributed in distinct regions, indicating significant differences in odor profiles among them. This demonstrates that the aroma characteristics of the samples vary considerably under different yeast fermentation conditions.

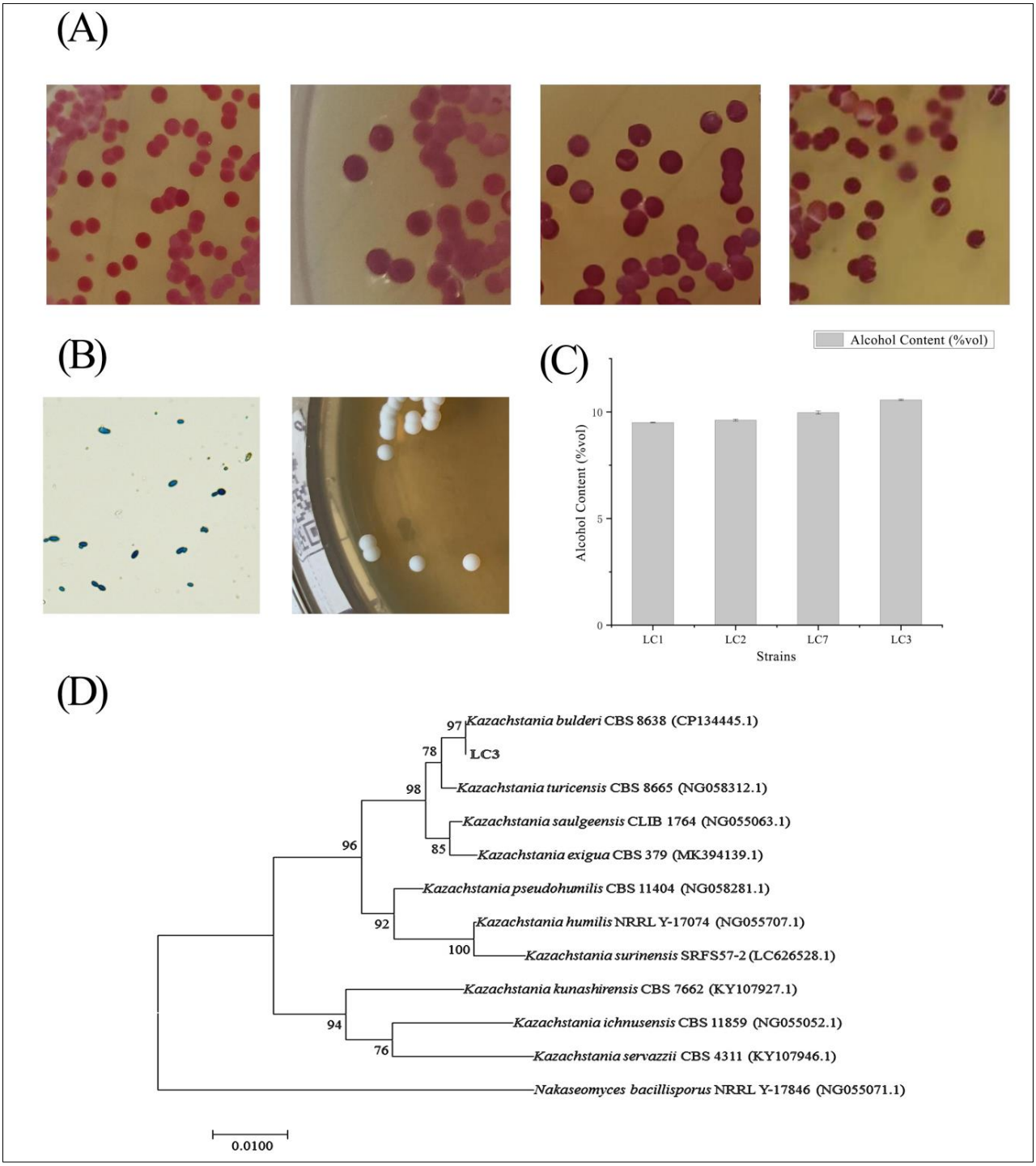

Fig. 1: Screening and identification results of strains

A. Color development results of four LC strains analyzed qualitatively by the TTC method, from left to right: LC1, LC2, LC3, LC7
B. Microscopic morphology and plate colony morphology of LC3 strain
C. Alcohol concentration of four LC strains after fermentation
D. Phylogenetic tree of LC3

The Electronic Nose flavor radar chart (Fig. 2C) shows that compared to Groups A and B, Group C exhibits significant changes in six sensors: W1C (aromatic compounds), W3C (ammonia and aromatic compounds), W5C (short-chain alkanes and aromatic components), W1S (methyl compounds), W2S

(alcohols, aldehydes, and ketones), and W3S (long-chain alkanes). In contrast, Group C shows lower responses in the W5S (nitrogen oxides) and W1W (sulfides) sensors compared to Groups A and B. The increase in aromatic compounds, alcohols, aldehydes, and ketones, coupled with the reduction in sulfides and nitrogen oxides, contributes to the superior electronic nose flavor profile of Group C compared to Groups A and B.

3.4. Flavor Wheel

After evaluation by the tasting panel, a total of 51 olfactory descriptors and 25 gustatory descriptors were collected for highland barley wine (Fig. 2D). These descriptors were preliminarily organized by merging terms with similar meanings, resulting in 37 olfactory descriptors and 15 gustatory descriptors. The 37 olfactory descriptors are: apple, banana, orange, pineapple, pomelo, lemon, pear, hawthorn, rose, jasmine, lilac, honey, honey aroma, cream, caramel, almond aroma, multi-grain aroma, cooked rice aroma, bean aroma, mellow aroma, qu (starter culture) aroma, cellar aroma, ester aroma, fermented grain aroma, aged aroma, sour aroma, aged storage vessel note, raw material note, rancid odor, metallic odor, earthy aroma, straw aroma, grassy aroma, woody aroma, fusel oil aroma, bran odor, and musty odor. The 15 gustatory descriptors are: refreshing, delicate, smooth and soft, spicy, bland, full-bodied, astringent, aftertaste, mellow and harmonious, lingering aftertaste, sour, sweet, bitter, salty, and umami.

In total, 52 flavor descriptors were obtained. With reference to the classification methods of foreign liquor flavor wheels reported in the literature and through discussion within the sensory evaluation panel, these flavor descriptors were categorized and summarized to establish the flavor wheel for highland barley wine.

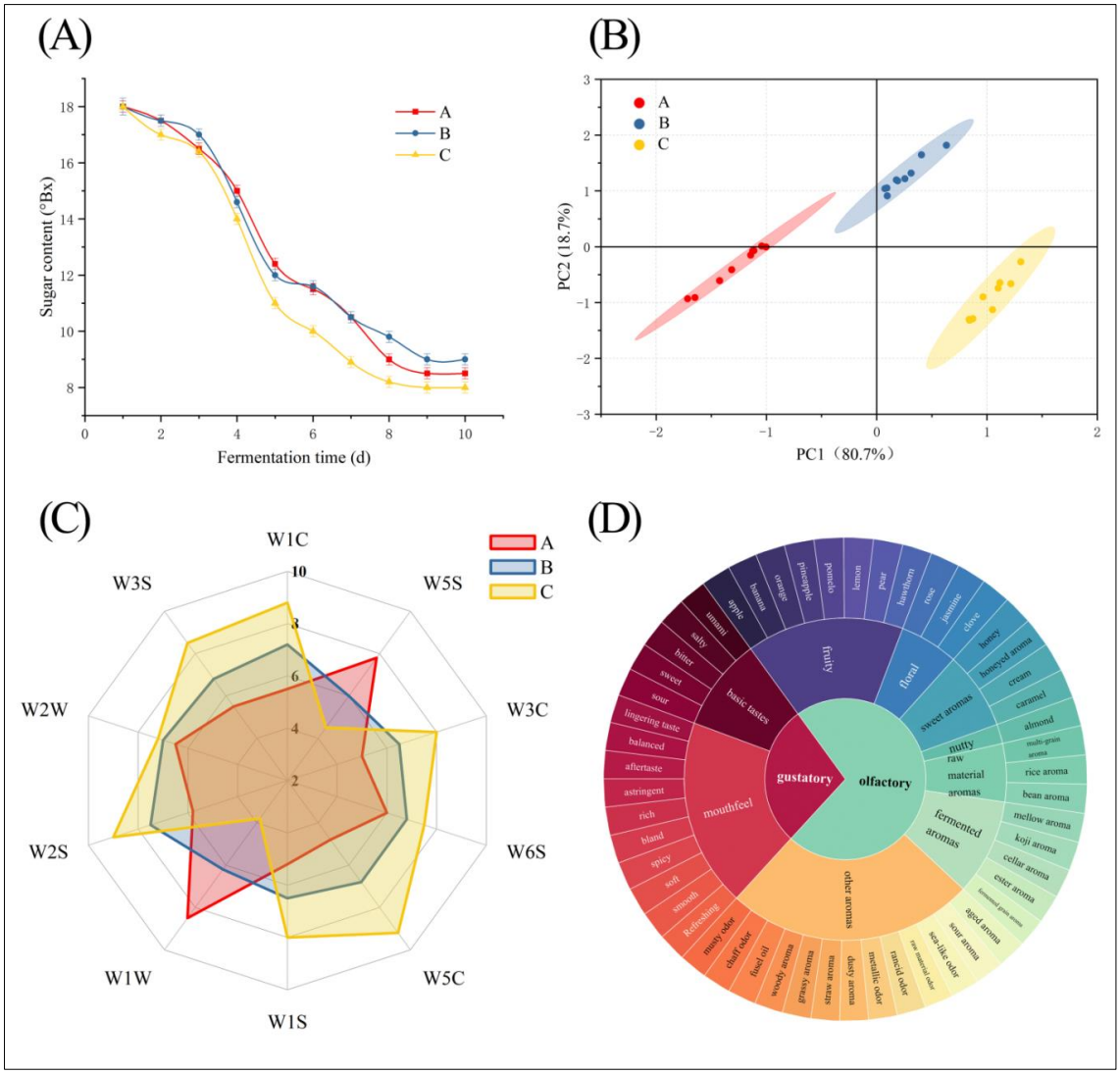

Fig. 2: Sugar content detection and flavor evaluation during fermentation (A: LC3 strain, B: commercial strain, C: mixed strain)

A. Changes in the sugar content of fermentation broths over time under identical conditions for LC3 strain, commercial strain, and mixed strain

B. PCA difference plot of three fermentation methods analyzed by electronic nose results

C. Electronic nose flavor radar charts under three fermentation conditions

D. Flavor wheel of highland barley whisky

3.5. GC×GC–TOFMS Volatile Profiling

Based on the results obtained from GC×GC-TOF MS, there were certain differences in the content of flavor compounds in the finished highland barley whisky between mixed-culture fermentation and single-culture fermentation using commercial yeast. Components with a match factor greater than 80 from the measurement results were retained and visualized in a heatmap (Fig. 3A). Group A represents the flavor compounds in the finished highland barley whisky produced by single-culture fermentation with LC3 yeast, Group B represents those from single-culture fermentation with commercial whisky yeast, and Group C represents those from mixed-culture fermentation using both commercial yeast W19 and LC3 yeast.

The heatmap shows that the differences in compound content between Group A and Group B were relatively small, whereas Group C exhibited significant overall differences compared to Groups A and B. This indicates that Group C had distinct variations in certain components, with their concentrations being higher than those in Groups A and B. A total of 63 volatile components with concentrations greater than 1 mg/L and match factors exceeding 80% were detected across the three whisky samples (Fig. 3B). These components provide a valuable reference for future research on volatile compounds in finished highland barley spirits. Among them, there were 8 alcohols, 44 esters, 4 aldehydes, 4 alkanes, 1 aromatic compound, and 2 ketones, as illustrated in the distribution chart. Among all aroma compounds, esters accounted for the highest proportion, followed by other categories including alkanes such as aromatic hydrocarbons, then alcohols, while ethers were the least abundant. The differences in aroma compounds among Groups A, B, and C were also primarily reflected in ester compounds.

Regarding ester compounds, the ethyl hexanoate content in Group C (0.7093 mg/L) increased by approximately 489% compared to Group A (0.1204 mg/L), while ethyl decanoate (6.1262 mg/L) reached over 15 times that of Group A (0.4039 mg/L). This substantial increase in esters significantly enhanced the fruity and floral layers of the whisky. Among higher alcohols, the isopropanol content in Group C (6.2585 mg/L) was 14 times that of Group A (0.4426 mg/L), and 3-methyl-1-butanol (1.3760 mg/L) was also significantly higher than in Group A (1.2989 mg/L), contributing greater body and complexity to the whisky. For aldehydes, the decanal content in Group C (5.8008 mg/L) increased by approximately 743% compared to Group A (0.6879 mg/L), while octanal (3.0052 mg/L) was more than 7 times that of Group A (0.4029 mg/L), notably enhancing the fresh and clean aroma of the whisky. Although Group B outperformed Group A in certain components, such as ethyl acetate (2.1500 mg/L vs. 0.3693 mg/L), its overall flavor profile remained significantly inferior to that of Group C.

In summary, the mixed-culture fermentation process, through synergistic effects, significantly increased the content and harmony of key flavor compounds in highland barley whisky, providing a reliable technical pathway for achieving superior product flavor.

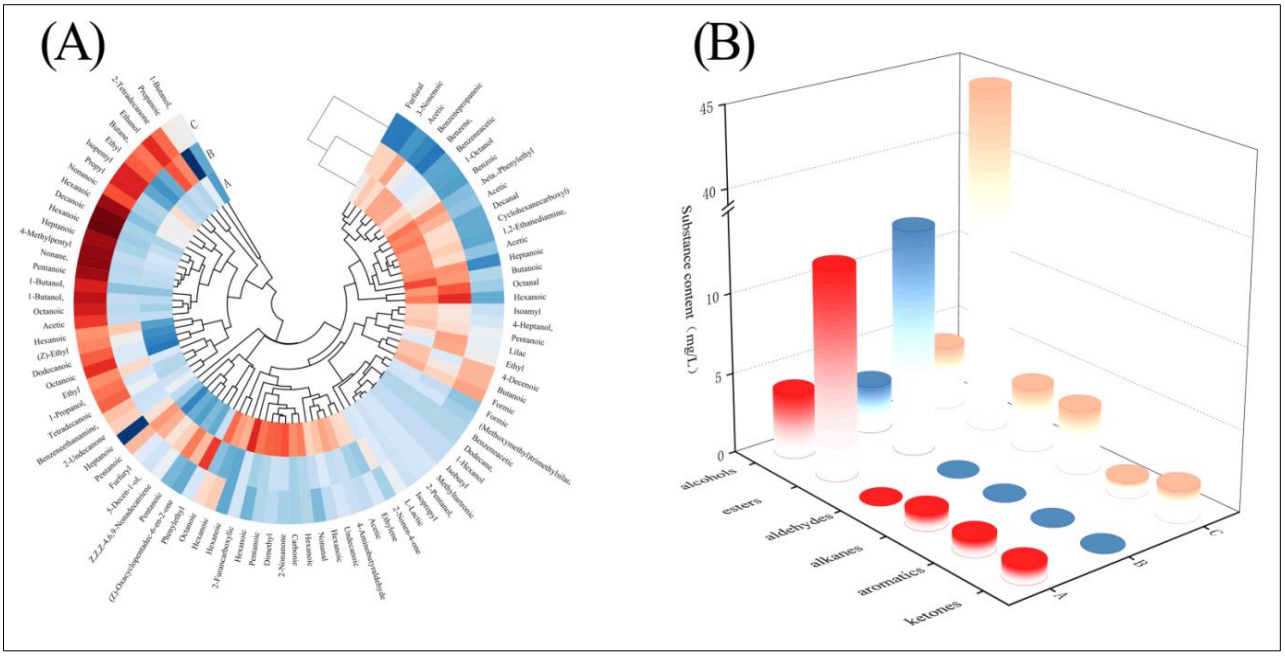

Fig. 3: GC×GC–TOFMS volatile profiling analysis results (A: LC3 strain, B: commercial strain, C: mixed strain)

A. Cluster heat map of comprehensive GC×GC–TOFMS
B. 3D plot of substance content from GC×GC–TOFMS

Continued Table: Concentration Results (mg/L) of 90 Common Volatile Components in Highland Barley Wine Analyzed

by GC×GC-TOFMS Technology

Number	CAS	Substance name	A	B	C
Alcohols（n=10）					
1	4265-97-8	Heptanoic acid, octyl ester	0.443	0.012	6.259
2	19329-89-6	Isoamyl lactate	0.080	0.000	0.352
3	25601-41-6	(Z)-Ethyl pentadec-9-enoate	2.721	0.010	0.786
4	76649-16-6	Ethyl trans-4-decenoate	0.112	0.335	0.008
5	626-82-4	Hexanoic acid, butyl ester	0.494	1.588	0.109
6	539-82-2	Pentanoic acid, ethyl ester	1.299	0.920	1.376
7	124-13-0	Octanal	0.586	1.583	0.895
8	35852-49-4	Hexanoic acid, 4-methylpentyl ester	0.327	1.775	1.775
9	10588-10-0	Pentanoic acid, 2-methylpropyl ester	1.404	0.600	0.600
10	626-77-7	Hexanoic acid, propyl ester	1.085	0.814	1.499
Esters（n=60）					
1	98-01-1	Furfural	0.001	0.065	3.334
2	60-12-8	Phenylethyl Alcohol	0.369	2.150	0.192
3	89353-62-8	Z,Z,Z-4,6,9-Nonadecatriene	2.054	0.091	0.036
4	63958-52-1	(Z)-Oxacyclopentadec-6-en-2-one	1.716	0.223	1.716
5	38421-90-8	(E)-5-DECEN-1-YL ACETATE	1.660	0.007	0.236
6	39252-02-3	Furfuryl hexanoate	3.099	0.315	0.076
7	103-52-6	.beta.-Phenylethyl butyrate	1.063	0.544	1.246
8	112-31-2	Decanal	0.943	0.040	1.734
9	103-45-7	Acetic acid, 2-phenylethyl ester	0.033	0.420	2.443
10	91213-30-8	3-Nonenoic acid, ethyl ester	0.134	0.057	3.385
11	112-12-9	2-Undecanone	0.040	0.242	0.091
12	105-76-0	Ethylene glycol di-n-butyrate	0.023	0.944	0.172
13	112-23-2	Formic acid, heptyl ester	0.212	0.407	1.070
14	101-97-3	Benzeneacetic acid, ethyl ester	0.366	0.086	1.947
15	111-87-5	1-Octanol	0.037	0.018	2.001
16	93-89-0	Benzoic acid, ethyl ester	0.122	0.000	2.126
17	32064-72-5	2-Nonen-4-one	0.173	0.000	0.754
18	122-43-0	Benzeneacetic acid, butyl ester	0.577	0.577	0.577
19	64-17-5	Ethanol	0.431	1.583	2.550
20	628-63-7	Acetic acid, pentyl ester	0.387	0.384	2.187
21	134-96-3	Lilac aldehyde C	0.000	0.437	0.055
22	76649-16-6	4-Decenoic acid, ethyl ester, (Z)-	0.141	0.104	0.250
23	1117-55-1	Octanoic acid, hexyl ester	3.544	0.086	2.466
24	106-33-2	Dodecanoic acid, ethyl ester	3.081	0.116	1.488
25	2345-27-9	2-Tetradecanone	0.578	0.734	1.247
26	54546-22-4	Ethyl 9-hexadecenoate	0.120	0.042	0.709
27	6290-37-5	Hexanoic acid, 2-phenylethyl ester	2.056	0.133	0.639
28	63169-61-9	Pentanoic acid, 2-methyl-, anhydride	1.217	0.521	0.227
29	141-78-6	Ethyl Acetate	0.454	2.534	1.460
30	97-64-3	Propanoic acid, 2-hydroxy-, ethyl ester	0.055	6.775	0.000
31	2050-95-5	1-Butanol, 3-methyl-, carbonate (2:1)	1.054	1.054	2.889
32	3843-03-3	Butane, 1,1-diethoxy-3-methyl-	0.658	1.741	1.049
33	2412-80-8	Pentanoic acid, 4-methyl-, methyl ester	0.481	0.481	4.718
34	123-29-5	Nonanoic acid, ethyl ester	0.007	0.764	1.680
35	2035-99-6	Octanoic acid, 3-methylbutyl ester	0.823	0.465	3.044
36	540-07-8	Hexanoic acid, pentyl ester	0.019	1.156	4.844
37	6378-65-0	Hexanoic acid, hexyl ester	0.404	0.867	6.126
38	78-83-1	1-Propanol, 2-methyl-	0.548	0.203	1.062
39	123-51-3	1-Butanol, 3-methyl-	0.501	0.406	3.837
40	2198-61-0	Isopentyl hexanoate	0.035	1.833	2.799
41	624-13-5	Propyl octanoate	0.027	0.568	2.804
42	35852-42-7	4-Methylpentyl 4-methylpentanoate	0.288	0.393	4.931

Number	CAS	Substance name	A	B	C
43	55429-85-1	PPCT	0.299	0.299	0.181
44	79-33-4	L-Lactic acid	0.109	0.109	0.040
45	112-14-1	Acetic acid, octyl ester	0.043	0.734	0.440
46	6117-97-1	Dodecane, 4-methyl-	0.569	0.569	0.569
47	123-92-2	1-Butanol, 3-methyl-, acetate	2.235	1.805	0.024
48	2051-49-2	Hexanoic acid, anhydride	0.912	0.576	0.576
49	106-32-1	Octanoic acid, ethyl ester	1.228	1.150	0.031
50	2050-09-1	Pentanoic acid, 3-methylbutyl ester	0.149	0.038	0.037
51	3289-28-9	Cyclohexanecarboxylic acid, ethyl ester	0.494	0.034	1.746
52	589-59-3	Isobutyl isovalerate	0.382	0.382	0.382
53	6314-97-2	Benzene, (2,2-diethoxyethyl)-	0.400	0.082	3.574
54	140-28-3	1,2-Ethanediamine, N-(phenylmethyl)-	0.535	0.108	1.867
55	112-06-1	Acetic acid, heptyl ester	0.721	0.263	1.062
56	142-92-7	Acetic acid, hexyl ester	0.531	0.034	1.097
57	123-66-0	Hexanoic acid, ethyl ester	0.972	2.518	1.708
58	4353-77-9	(Methoxymethyl)trimethylsilane	0.230	0.222	0.843
59	627-90-7	Undecanoic acid, ethyl ester	0.409	0.395	0.157
60	115-10-6	Dimethyl ether	1.419	0.297	0.511
Aldehydes（n=8）					
1	2021-28-5	Benzenepropanoic acid, ethyl ester	0.403	0.010	3.005
2	629-33-4	Formic acid, hexyl ester	0.320	0.069	0.453
3	124-06-1	Tetradecanoic acid, ethyl ester	0.599	0.032	1.377
4	110-38-3	Decanoic acid, ethyl ester	0.688	0.673	5.801
5	109-25-1	Heptanoic acid, 3-methylbutyl ester	0.209	0.690	4.711
6	34860-03-2	2-Pentanol, 3-methyl-, 2-acetate	0.224	0.224	0.224
7	105-88-8	Hexanoic acid, 2-methylpropyl ester	0.157	0.113	0.453
8	108-82-7	4-Heptanol, 2,6-dimethyl-	0.099	0.009	0.188
Alkanes（n=4）					
1	54815-13-3	Nonane, 1,1-diethoxy-	0.402	0.555	5.035
2	821-55-6	2-Nonanone	1.674	0.371	0.753
3	67-63-0	Isopropyl Alcohol	0.287	0.287	0.287
4	124-19-6	Nonanal	0.062	0.413	1.293
Aromatics（n=3）					
1	111-27-3	1-Hexanol	0.382	0.382	0.382
2	595-98-2	Methyltartronic acid	0.191	0.191	0.191
3	614-99-3	2-Furancarboxylic acid, ethyl ester	2.710	0.222	0.222
Ketones（n=5）					
1	6346-09-4	4-Aminobutyraldehyde diethyl acetal	0.289	0.341	0.341
2	105-54-4	Butanoic acid, ethyl ester	0.147	0.000	0.228
3	106-27-4	Butanoic acid, 3-methylbutyl ester	1.754	0.573	0.583
4	106-30-9	Heptanoic acid, ethyl ester	0.677	0.619	2.796
5	7507-16-6	Carbonic acid, ethyl nonyl ester	0.709	0.843	0.843

4. CONCLUSION AND FUTURE PERSPECTIVES

Highland barley whisky, a distilled spirit primarily made from highland barley and malt, derives its flavor profile mainly from the raw materials and volatile compounds produced during microbial metabolism in the fermentation process. Its typical flavor is characterized by a rich and sweet base, with its quality heavily dependent on the types, concentrations, and balance of esters, higher alcohols, aldehydes, and trace aromatic substances generated during fermentation. Among these, esters are the key contributors to fruity and floral notes; higher alcohols, in appropriate amounts, enhance the body and complexity of the whisky, while excessive levels may lead to off-flavors; aldehydes and similar compounds contribute fresh and clean aromas. Therefore, fermentation technology, particularly the selection and combination of yeast strains, is the core factor in shaping and enhancing the flavor quality of highland barley whisky.

This study systematically compares the flavor contributions of commercial yeast and wild yeast strains isolated from fermented pit mud used in baijiu production during the fermentation of highland barley whisky. It focuses on the impact of single-strain and mixed-strain fermentation on the flavor composition and

sensory quality of the spirit. Through multi-dimensional analytical methods, including electronic nose, human sensory evaluation, and comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC×GC–TOF MS), the study reveals the variations in the composition and concentration of flavor compounds under different fermentation approaches.

The experimental results indicate that the yeast strain LC3, isolated from baijiu pit mud, exhibited superior flavor characteristics compared to W19 in single-strain fermentation, with a notable advantage in the synthesis of ester compounds. Further research found that mixed fermentation using LC3 and W19 significantly increased the variety and concentration of key flavor compounds in the spirit. The increase in esters was particularly pronounced; for instance, the concentrations of ethyl hexanoate and ethyl decanoate increased by approximately 489% and over 15 times, respectively, compared to the group fermented with the commercial yeast alone. These compounds play a crucial role in forming the fruity and floral notes of the whisky.

Additionally, mixed fermentation promoted the accumulation of higher alcohols (such as isopropanol and 3-methyl-1-butanol) and aldehydes (such as decanal and octanal), further enhancing the body and fresh aromas of the spirit. Electronic nose analysis also supported the superiority of mixed fermentation samples in overall odor profiles, showing more significant responses in aromatic, alcoholic, and aldehyde/ketone compound categories, while the levels of undesirable odor components such as sulfides and nitrogen oxides were relatively reduced.

In summary, mixed fermentation, through synergistic metabolic interactions between yeast strains, not only enriches the flavor layers of highland barley whisky but also effectively improves its overall aromatic harmony and sensory acceptability. Starting from microbial screening and combination strategies, this study provides a feasible technical approach and theoretical foundation for enhancing the quality of highland barley-based spirits. It also offers new perspectives on integrating traditional brewing ingredients with modern fermentation technologies. Future research could further explore the metabolic interaction mechanisms of different yeast combinations to optimize fermentation processes and achieve precise flavor control.

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