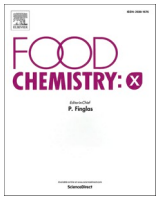
Food Chemistry: X 25 (2025) 101952

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Sequential bioaugmentation of the dominant microorganisms to improve the fermentation and flavor of cereal vinegar

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ARTICLE INFO

Keywords:
Cereal vinegar
Solid-state fermentation
Sequential bioaugmentation
Flavor quality
Lactobacillus helveticus
Acetobacter pasteurianus

ABSTRACT

Traditional cereal vinegars are fermented by microorganisms that are spontaneously enriched, leading to uncertainty in regulating the fermentation process and flavor. The objective of this study was to elucidate the impact of the predominant microorganisms, provenly *Lactobacillus helveticus* and *Acetobacter pasteurianus*, on the solid-state fermentation (SSF) and flavor profile of cereal vinegar by several bioaugmentation strategies. The results indicated that the sequential bioaugmentation of predominant microorganisms improved the utilization of raw material and most key flavor compounds. Through sequential bioaugmentation strategy, bacterial diversity was regulated due to minimizing acetic acid inhibition in the early stages, and the non-volatile acid was targetedly improved by *Lactobacillus*. Furthermore, the important flavor of non-volatile acid, esters, acetoin, and tetramethyl-pyrazine content was enhanced by sequential bioaugmentation. Therefore, the sensory score on taste and odor were improved. These results provide a reference for the targeted regulation of the SSF and the flavor quality of cereal vinegar.

1. Introduction

Vinegar, a time-honored condiment, is predominantly produced through the fermentation of cereals or fruits. In 2020, the global vinegar market registered a valuation of $67.3 billion, and it is anticipated to experience substantial growth, reaching an estimated $103.9 billion by 2026, exhibiting a robust compound annual growth rate of 6.3 % (Statista, 2022). In China, traditional vinegar plays a crucial role in Chinese culture and daily life. Many types of Chinese vinegar are produced by spontaneous solid-state fermentation (SSF) technology with cereals as raw materials, including sorghum, rice, and bran. The process of alcohol fermentation and acetic acid fermentation (AAF) are essential phases in the making of cereal vinegar (Zhang et al., 2020). In particular, the AAF is the decisive phase in vinegar flavor compound formation (Wu et al., 2021). Hundreds of microorganisms take part in the AAF, e.g. lactic acid bacteria (LAB), acetic acid bacteria (AAB), *Bacillus*, etc. In particular, *Lactobacillus* and *Acetobacter* are the predominant microorganisms for cereal vinegar fermentation (Zhang et al., 2020). Traditionally, AAF of cereal vinegar is mainly produced by SSF in a pottery vat (Ye et al., 2023). To improve the mass transfer, turning over *Cupei* (a mixture of fermentation materials) is performed every day, and consumes a lot of labor resources. In recent years, with the progress of mechanization, the traditional production mode (pottery vat and manual) is transitioning towards a mechanized process (fermented pool and mechanized) to reduce labor requirements. However, challenges such as fluctuating quality and long fermentation times persist during the shift to mechanization upgrades in the SSF of vinegar. Therefore, it is urgent to resolve these problems in the SSF of cereal vinegar.

Bioaugmentation has been proven as an effective means to improve the fermentation process of traditional fermented foods, and to regulate the microbial composition, ultimately enhancing the flavor quality (Chantarot et al., 2022; Kilic et al., 2022; Padilla et al., 2017). Previous studies have identified LAB and AAB as the dominant microorganisms in fermented food by high-throughput sequencing technology (Bationo et al., 2023), and these microorganisms have been applied in vinegar production to enhance production efficiency (Zhang et al., 2021), as

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https://doi.org/10.1016/j.fochx.2024.101952
Received 31 August 2024; Received in revised form 29 October 2024; Accepted 30 October 2024
Available online 1 November 2024

well as to improve the flavor and quality of vinegar (Tanamool et al., 2020). Furthermore, LAB plays an essential role in flavor formation, including lactic acid and malic acid, and in synthesizing 2,3-butanediol (Maske et al., 2024). The lactic acid produced by LAB makes the vinegar taste softer (Sun et al., 2023). AAB has the ability to convert ethanol into acetic acid and to produce acetoin (Moens et al., 2014). Acetoin is a precursor of tetramethyl-pyrazine (TMP), which contributes to the creamy odor of vinegar (Sun et al., 2023). TMP was a functional compound in cereal vinegar and provided a nutty odor (Tian et al., 2023). Regulating the content of acetoin and TMP is a potent strategy to improve the flavor quality of cereal vinegar. In addition, LAB and AAB are also widely used for short fermentation time and improve quality in other fermented foods, including soy sauce and fermented vegetables (de Castro et al., 2019; Devanthi et al., 2018), etc. These results could provide adequate information about bioaugmentation in fermented food. However, LAB and AAB have ecological relations of amensalism in cereal vinegar fermentation (Xia et al., 2022). The mono-bioaugmentation of LAB can affect the fermentation temperature and oxygen levels in *Cupei* (Zhang et al., 2020), whereas mono-bioaugmentation of *Acetobacter* could affect microbial diversity due to high acetic acid inhibition (Xia et al., 2022), further impacts the taste of vinegar. Consequently, studies on bioaugmentation strategies of SSF in cereal vinegar are necessary to improve flavor quality by regulating bacterial profiles.

In our previous study, *Acetobacter pasteurianus* and *Lactobacillus helveticus* were proven the predominant species of AAB and LAB, respectively, and they showed negative interrelationship during the SSF of cereal vinegar (Xia et al., 2022). Here, we investigated the effect of several different bioaugmentation strategies of the dominant microorganism (*L. helveticus* and *A. pasteurianus*), with a specific focus on flavor regulation. This study could provide a theoretical framework for enhancing the transformation of raw materials and the quality of vinegar.

2. Materials and methods

2.1. Preparation of strains

L. helveticus CGMCC 12062 and *A. pasteurianus* CGMCC 3089 were isolated from traditional cereal vinegar fermentation (Shanxi Zilin Vinegar Industry Co., Ltd., Taiyuan, China) and were preserved in the Chinese General Microbiological Culture Collection Center. *L. helveticus* CGMCC 12062 was activated and sub-cultured in a 5 L fermenter (BXBIO, Shanghai, China) containing 3 L of MRS broth for 24 h at 37 °C ($OD_{600\ nm} = 2.0$). *A. pasteurianus* CGMCC 3089 was activated and sub-cultured in a Frings 8 L Pilot-Acetator (Heinrich Frings GmbH & Co KG, Rheinbach, Germany) containing 5 L of GY broth at 30 °C and 200 rpm for 24 h ($OD_{600\ nm} = 0.8$). Subsequently, the collected cells were resuspended in sterile 0.9 % (*w/v*) saline and employed for inoculation fermentation. The inoculation cell density of *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 was adjusted to approximately 10^6 and 10^5 CFU/g *Cupei*, respectively. Five experimental batches were prepared, including (1) Control (spontaneous fermentation, C group), (2) Mono-bioaugmentation with *L. helveticus* CGMCC 12062 (L group), (3) Mono-bioaugmentation with *A. pasteurianus* CGMCC 3089 (A group), (4) Co-bioaugmentation with *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 (L+A group), (5) Sequential bioaugmentation with *L. helveticus* CGMCC 12062 (inoculated at 0 days) and *A. pasteurianus* CGMCC 3089 (inoculated at 5 days) (L-A group).

2.2. Solid-state fermentation and sample collection

Bioaugmented experiments on SSF of cereal vinegar were conducted in the fermentation plant of Shanxi Zilin Vinegar Co., Ltd. (Taiyuan, Shanxi province, China). The samples of *Cupei* were collected at designated time points: 0, 1, 5, 9, 13, and 17 days, representing the initial, early, middle-early, middle, middle-late, and late stages of fermentation, respectively. The samples were put into sterile and stored at −80 °C for subsequent analysis.

2.3. Physicochemical properties analysis

2.3.1. pH, total titratable acidity, ethanol content, non-volatile acid, amino nitrogen, temperature, reducing sugar, and the utilization ratio of starch raw materials

The pH, total titratable acidity (TTA), amino nitrogen, and non-volatile acid were measured according to the method of the previous study (Sun et al., 2023). The ethanol content was detected by a biosensor (SBA—40C, Shan Dong Academy of Sciences, Jinan, China). The reducing sugar content was measured by the 3,5-dinitro salicylic acid (DNS) method of the previous study (Huang et al., 2022). The utilization ratio of starch raw materials was determined by the previous study, and the calculation formula is shown in formulation (1) (Zhang et al., 2021). The fermentation temperature was determined by a temperature sensor (L93–3, Hangzhou Loggertech Co., Ltd., Hangzhou, China).

$$\text{Starch utilization} = \frac{\text{total titratable acid in } \textit{Cupei} + \text{ethanol content in } \textit{Cupei} \times 1.304}{(\text{total raw starch} - \text{total sugar in } \textit{Cupei} \times 0.9) \times 0.740} \quad (1)$$

2.3.2. Total phenolic content, total flavonoid content, organic acid, and volatile flavor compounds

The total phenolic content (TPC) and total flavonoid content (TFC) were detected by the previous method according to the previous study (Zhang et al., 2023). High-performance liquid chromatography (HPLC) was used to detect five main organic acids (acetic acid, lactic acid, citric acid, malic acid, and oxalic acid) according to the method of the previous study (Zhang et al., 2021). Briefly, the eluent consisted of 5 mmol/L H_2SO_4. The mobile phase was set at 0.6 mL/min, the column oven was maintained at 30 °C and the detected wavelength of the UV detector was 215 nm. The volatile flavor compounds (VFCs) of *Cupei* were detected by GC–MS 8890-7000D (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a column of DB-WAX, 30 m × 0.25 mm, 0.25 μm (column length × inner diameter, film thickness) (Wang et al., 2024).

2.4. Microbial diversity analysis

The DNA of *Cupei* was extracted according to the method of the previous study (Huang et al., 2022). The quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR amplification of the bacterial 16S rRNA genes V3-V4 region was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μl of buffer (5×), 0.25 μl of Fast pfu DNA Polymerase (5 U/μl), 2 μl (2.5 mM) of dNTPs, 1 μl (10 uM) of each Forward and Reverse primer, 1 μl of DNA Template, and 14.75 μl of ddH2O. Thermal cycling consisted of initial denaturation at 98 °C for 5 min,

followed by 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 5 min at 72 °C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads(Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, sequencing was performed using the Illlumina NovaSeq 6000 platform with PE250 strategy at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The sequence analysis is shown in Appendix.

2.5. Sensory evaluation

Sensory evaluation was conducted with 10 trained evaluators (5 males and 5 females) between the ages of 20 and 50. These evaluators were recruited from Tianjin University of Science and Technology. The evaluators were instructed to rinse their mouths with water for about 60 s for each sample. The criteria for the sensory evaluation of cereal vinegar are listed in Table S2 according to the Chinese national standard GB/T 19777–2013 with some modifications. Briefly, a total of four descriptors of color, posture, odor, and taste were evaluated. The score of intensity ratings ranged from 1 to 10.

2.6. Statistical analysis

All experiments were repeated three times independently. The data were statistically analyzed using IBM SPSS Statistics 26 (IBM, New York, USA) software. The graphs were plotted using Origin 2022b (OriginLab Corporation, MA, USA). Redundancy analysis (RDA) was conducted using Canoco for Windows v4.5 (Wageningen UR, Netherlands). All data are expressed as the mean values or mean ± standard deviation.

3. Results

3.1. Analysis of bacterial community composition

Illumina HiSeq sequencing generated 1,953,217 raw sequence reads from all samples. After undergoing quality control processing including filtering, denoising, merging, and removing chimeric and singleton sequences, a total of 1,428,608 high-quality tags were acquired. On average, each sample was represented by 476,20 tags (Table 1). The rarefaction curves for all samples reached a saturation plateau (Fig. S1) with coverage exceeding 0.99 (Table 1), suggesting that the majority of bacterial phylotypes present in the samples were sufficiently captured. The alpha diversities of the bacterial community in *Cupei* samples are detailed in Table 1. The total number of observed species (at 97 % sequence similarity level) for bacteria ranged from 57.60 to 588.20. The number of observed species in bioaugmented samples exhibited a fluctuating trend, firstly decreasing and then increasing during fermentation, whereas the number of observed species in the C group gradually declined (Table 1). The highest Chao1 indices were observed in the L-A group indicating that the species richness is the highest in the L-A group. The change tendency of Chao1 indices and Observed species were similar. Furthermore, the Shannon and Simpson indices provided insights into the approximate number of ASVs and the distribution evenness within the samples, as displayed in Table 1.

The bacterial community in all *Cupei* samples encompassed 10 phyla, including Firmicutes, Proteobacteria, Actinobacteriota, Bacteroidota, Verrucomicrobiata, Deinococcota, Planctomycetota, Desulfobacterota, Acidobacteriota, Choroflexi, and Others. Over 90 % of the annotated reads were attributed to Firmicutes and Proteobacteria (Fig. 1A). Firmicutes (53.46 %–95.16 %) exhibited a first increase followed by a decrease, and dominated in all five group treatments at the end of fermentation. On the other hand, Proteobacteria (4.14 %–49.11 %) demonstrated a decline and subsequent increase during fermentation, ultimately prevailing in the late stages of the fermentation. As shown in

Table 1
Alpha diversity of the bacterial community in cereal vinegar fermented by *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089.

Sample	Chao1	Shannon	Simpson	Observed species	Coverage
C_0	612.76	6.26	0.97	533.30	0.9897
C_1	270.52	3.86	0.82	236.30	0.9958
C_5	102.87	2.72	0.71	83.20	0.9983
C_9	82.17	3.18	0.85	72.40	0.9986
C_13	89.06	2.64	0.79	65.70	0.9982
C_17	74.00	2.19	0.75	57.60	0.9984
L_0	432.60	4.87	0.88	375.30	0.9923
L_1	277.92	4.41	0.87	237.90	0.9952
L_5	131.33	3.52	0.85	106.80	0.9977
L_9	116.57	2.88	0.77	91.60	0.9978
L_13	126.50	2.43	0.74	99.00	0.9974
L_17	189.70	3.55	0.82	157.30	0.9971
A_0	679.68	6.13	0.95	588.20	0.9879
A_1	414.98	5.13	0.94	361.80	0.9928
A_5	162.41	4.11	0.91	138.00	0.9974
A_9	131.13	3.51	0.84	99.80	0.9976
A_13	143.20	3.15	0.81	118.70	0.9976
A_17	142.95	2.97	0.79	107.30	0.9971
L+A_0	423.41	5.81	0.94	422.70	0.9992
L+A_1	378.69	4.75	0.92	339.20	0.9936
L+A_5	135.52	3.88	0.90	106.50	0.9977
L+A_9	127.76	3.49	0.86	98.40	0.9977
L+A_13	125.49	3.02	0.81	93.80	0.9976
L+A_17	185.18	3.53	0.85	146.90	0.9966
L-A_0	449.74	5.58	0.89	440.20	0.9967
L-A_1	344.85	4.76	0.92	281.30	0.9936
L-A_5	164.00	3.92	0.89	133.50	0.9972
L-A _9	145.21	3.05	0.79	118.30	0.9973
L-A_13	133.45	2.92	0.79	110.30	0.9978
L-A_17	191.49	3.48	0.83	149.00	0.9968

Fig. 1B, the genus, including *Lactobacillus*, *Acetobacter*, and *Limosilactobacillus* represented the predominant bacterial genus in five group treatments. The relative abundance of *Lactobacillus* and *Acetobacter* in five groups (C, L, A, L+A, and L-A) was 34.42 %, 47.90 %, 32.87 %, 34.59 %, 46.96 %, and 6.13 %, 10.43 %, 9.72 %, 13.19 %, 11.72 %, respectively, at the initial stage of fermentation. Bioaugmentation by *L. helveticus* CGMCC 12062 enhances the relative abundance of *Lactobacillus*, while bioaugmentation by *A. pasteurianus* CGMCC 3089 reduces the relative abundance of *Lactobacillus* due to the ecological relations of amensalism between *Lactobacillus* and *Acetobacter* (Xia et al., 2022). During the fermentation, the relative abundance of *Lactobacillus* was increased and then decreased in bioaugmentation groups, while it decreased then increased in the C group. *Limosilactobacillus* predominated at the early and middle-early stages of fermentation in the C group, while *Lactobacillus* predominated at all stages of fermentation in bioaugmented groups. Different bioaugmentation strategies both reduce the relative abundance of *Limosilactobacillus* compared with C group. *Acetobacter* predominated at the middle and late stages of fermentation in five group treatments, especially the relative abundance of inoculating *Acetobacter* (A group, L+A group, and L-A group) were obviously higher than C group and L group at the later stage. Furthermore, hierarchical clustering analysis indicated that samples were divided into two groups (Fig. 1C). The middle, middle-late, and late stages of bioaugmented groups were divided into one category, and other samples were split into another category. The structure of the microbiota in different bioaugmentation strategies at the genus level are similar in the late stage of fermentation.

3.2. Dynamics of physicochemical parameters

The change in physicochemical properties including pH, TTA, ethanol, amino nitrogen, reducing sugar, non-volatile acid, temperature, and the utilization ratio of starch raw materials are shown in Fig. 2. Low pH and high TTA were observed in bioaugmented samples (Fig. 2A-B). The average acid production rate of the C group was 0.1441 g/100 g

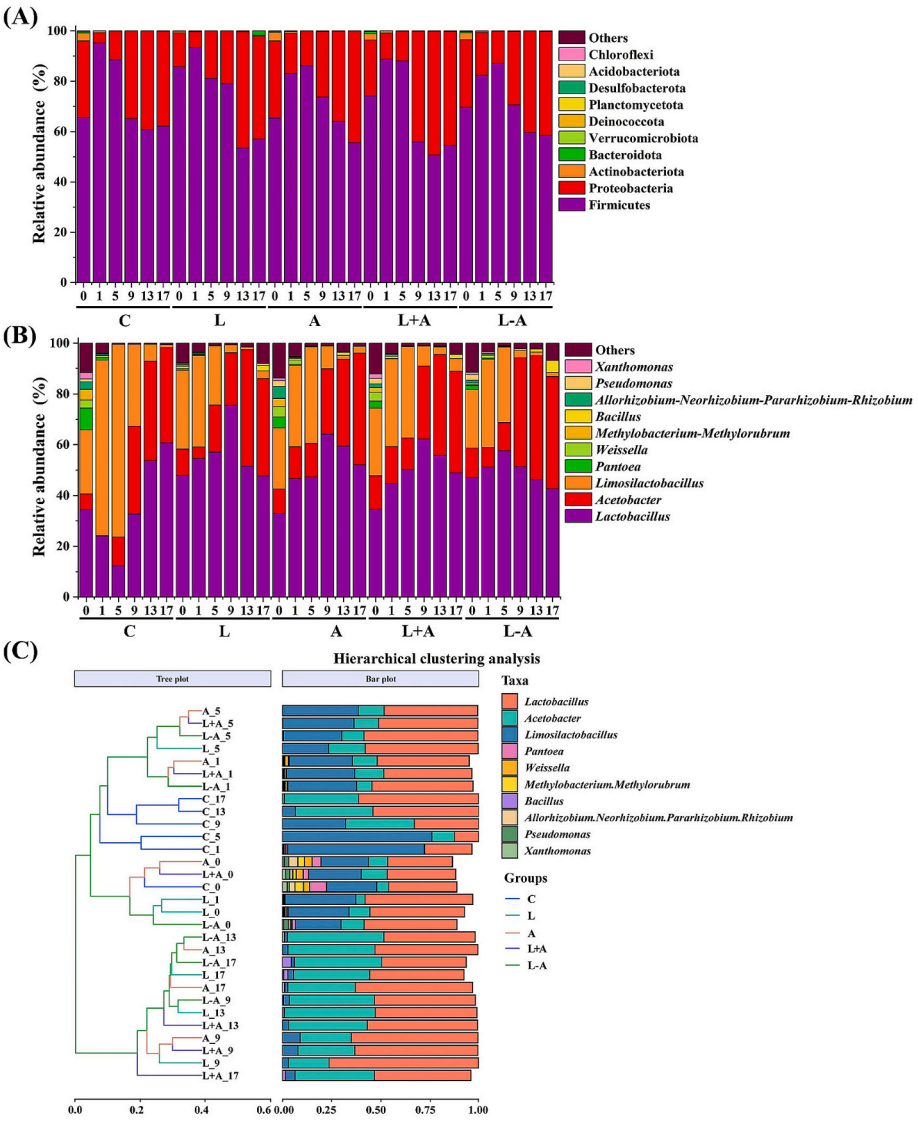

Fig. 1. The relative abundance of bacterial communities during the fermentation of cereal vinegar by inoculating *L. helveticus* and *A. pasteurianus* at the phylum level (A) and genus level (B). Hierarchical clustering analysis (C) of the top 10 genus of bacteria according to genus abundant information.

Cupei, while the average acid production rate of bioaugmentation groups of L, A, L+A, and L-A groups were 0.1726, 0.1846, 0.1944, and 0.1812 g/100 g *Cupei*/day, respectively. The production intensity enhanced by 19.78 %, 28.11 %, 34.91 %, and 25.74 %, respectively, compared with the C group. The ethanol utilization of the five groups (C, L, A, L + A, L-A) was 73.88 %, 77.43 %, 83.24 %, 85.84 %, and 84.99 %, respectively (Fig. 2C). The ethanol utilization was approximately enhanced by 3.55 %, 9.36 %, 11.96 %, and 11.11 %, respectively, compared with that of C group. As shown in Fig. 2D-E, the content of non-volatile acid and amino nitrogen contents both increased and then decreased during the fermentation in five groups. The L group had the highest non-volatile acid which was approximately increased by 13.96 %, compared with the C group. However, the highest amino nitrogen content was observed in the L+A group at the end of fermentation. On the whole, the changing trend of reducing sugar content under different bioaugmentation strategies was roughly the same, except for the L+A group (Fig. 2F). The content of reducing sugar in the L+A group was decreased and then increased during the fermentation. Besides, the lowest reducing sugar content was observed in samples of the L group at the end of fermentation. There is no temperature control facility in the SSF process of vinegar, thus biological heat was produced and accumulated due to the microbial metabolism. The change in fermentation temperature and the utilization of starch in five group treatments are shown in Fig. 2G-H. The temperature of the C group gradually increased, while they increased and then decreased in all bioaugmentation groups. The temperature increased rapidly to 40.2 °C on day one of the L+A group indicating enhanced microbial metabolism, while other groups were lower than 39 °C. The temperature changes in *Cupei* are related to microbial metabolism and the specific surface area of fermenters in the SSF of cereal vinegar. However, in the present study, the specific surface area in different strategies was similar, the temperature was mainly affected by microbial metabolism. Furthermore, different bioaugmentation strategies boosted the utilization of starch from 61.61 % (C group) to 65.87 % (L group), 64.72 % (A group), 69.88 % (L+A group), and 68.94 % (L-A group), respectively.

The relation among 16S_ASVs and physicochemical properties was analyzed by the Mantel test (Fig. 3A). These results indicated that 16S_ASVs had a positive association with pH and temperature. There was an obvious relation between 16S_ASVs and TTA ($r = 0.6$, $p < 0.01$), ethanol ($r = 0.6$, $p < 0.01$), and amino nitrogen ($r = 0.6$, $p < 0.01$). Therefore, the bacterial community of cereal vinegar could be optimized by controlling environmental parameters. Besides, TTA was positively related to temperature and amino nitrogen, while it was negatively correlated to ethanol and reducing sugar. Furthermore, the bacterial

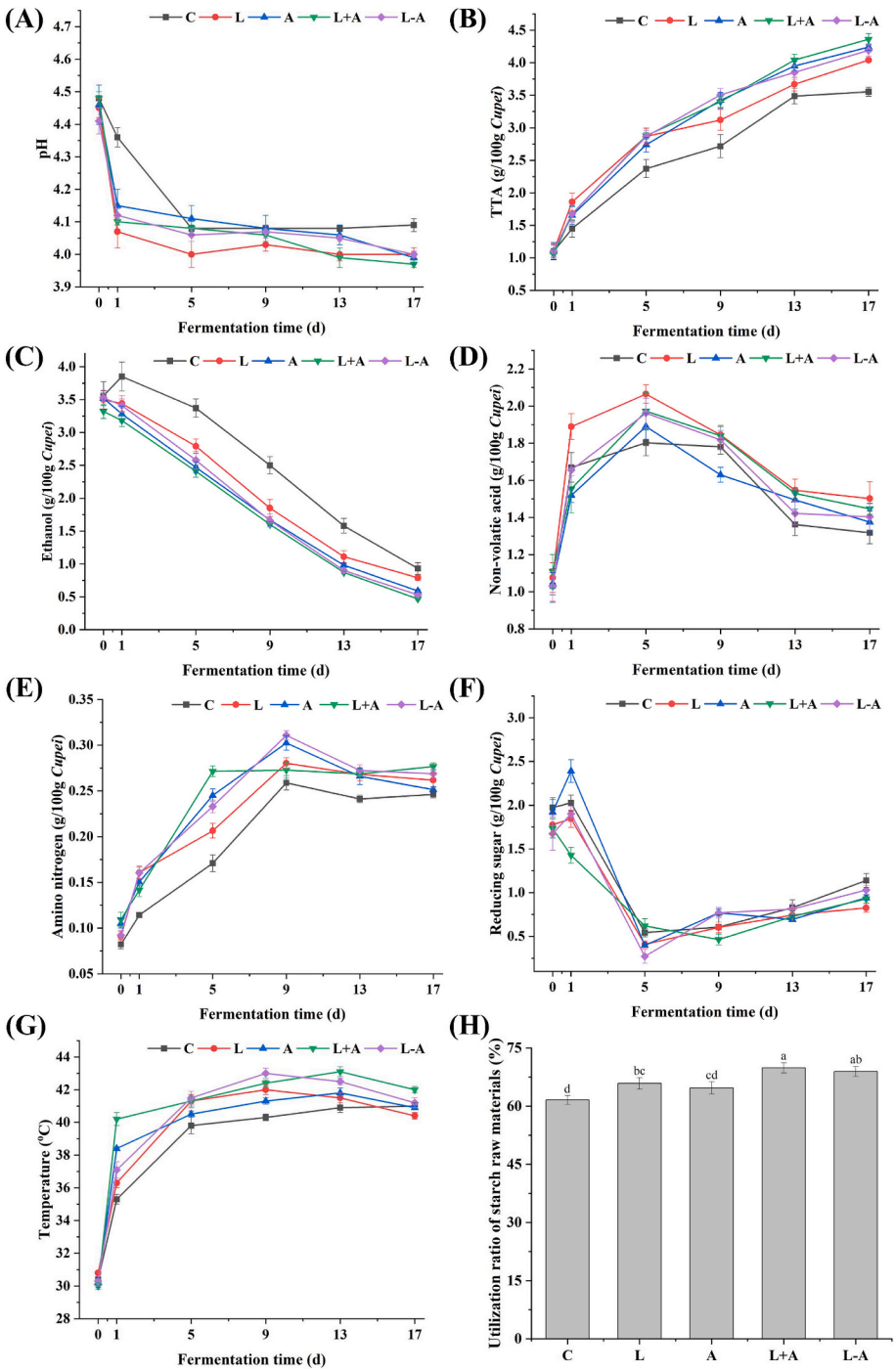

Fig. 2. Dynamics of physicochemical parameters. pH(A); TTA (B); Ethanol (C); Non-volatile acid (D); Amino nitrogen (E); Reducing sugar (F); Temperature (G); Utilization ratio of starch raw materials (H).

community structure was regulated by bioaugmentation strategies, which indirectly affected the physicochemical properties of cereal vinegar during the fermentation.

As shown in Fig. 3B, the correlation between the main genus and physicochemical properties during the cereal vinegar fermentation process was analyzed via RDA. The first two canonical axes accounted for 71.46 % and 8.89 % of the variation, demonstrating a notable correlation between bacteria and physicochemical properties. According to RDA analysis, the bacterial community was influenced by reducing sugar, ethanol, temperature, and TTA. TTA showed a positive correlation with the late stage of fermentation, whereas non-volatile acid was positively correlated with the middle and middle-late stages of fermentation. *Lactobacillus*, *Acetobacter*, and *Bacillus* were clustered in the third quadrant and showed correlations with TTA, amino nitrogen, and temperature. Furthermore, *Limosilactobacillus*, *Methylobacterium-Methylorubrum*, *Weissella*, *Pantoea*, *Xanthomonas*, *Pseudomonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, Others, etc., were correlated with the early and middle-early stage of the fermentation of cereal vinegar. These results are consistent with those of other studies (Huang et al., 2022; Sun et al., 2023).

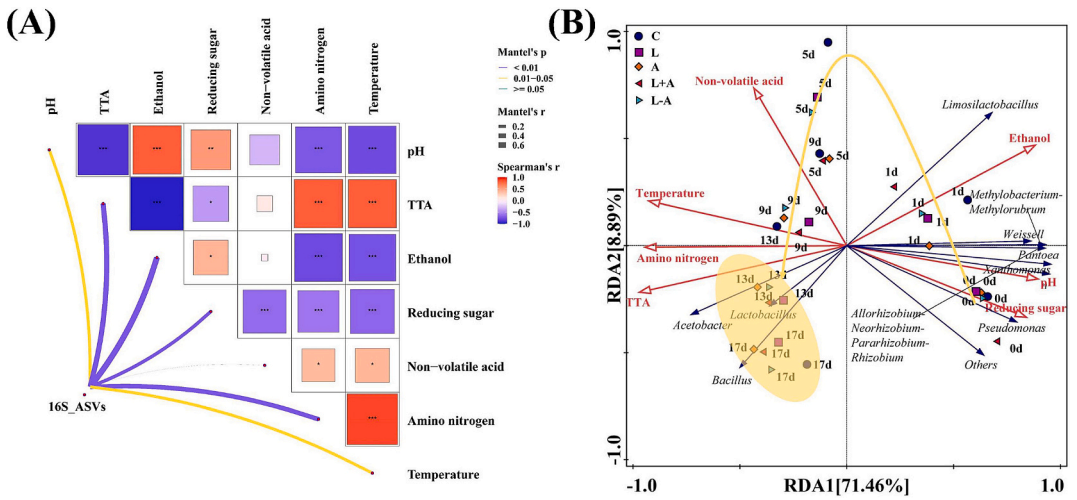

Fig. 3. The correlation between 16S_ASV and physicochemical property (A). The redundancy analysis of bacterial community during fermentation (B).

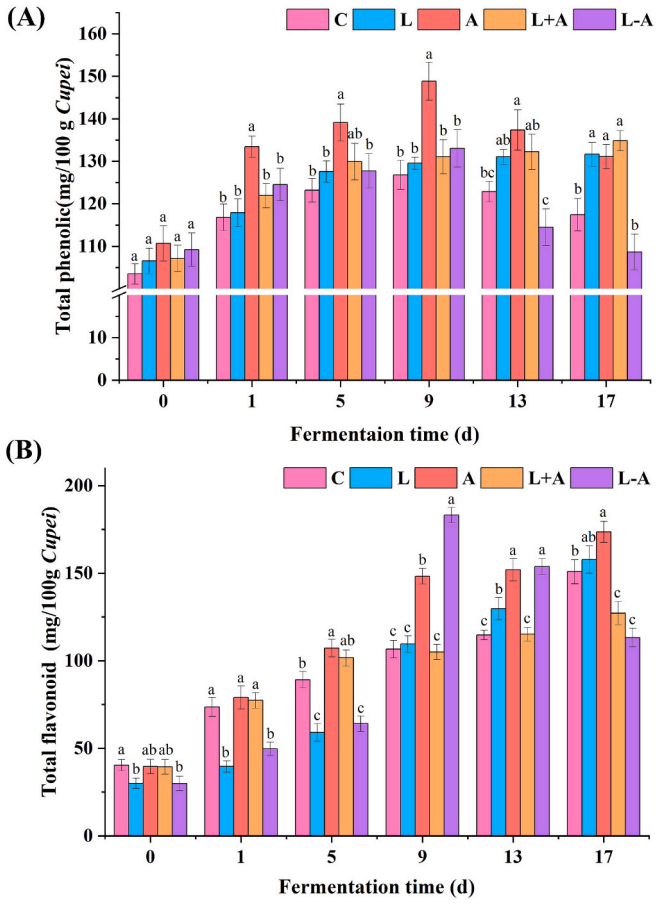

Fig. 4. Changes in total phenolic content (A), and total flavonoid content (B) during the fermentation.

3.3. Change in total phenolic content, total flavonoid content, and organic acids content

The TPC in the C group ranged from 103.51 to 123.18 mg/100 g *Cupei*, while it varied from 106.56 to 148.85 mg/100 g *Cupei* in the bioaugmented groups (Fig. 4A). The TPC of the C group, A group, and L-A group initially increased and then decreased. However, the TPC in the L group and L+A group gradually increased during the fermentation. The TPC of bioaugmented groups had no obvious difference, and was significantly higher than that of the C group at the end of fermentation, except for the sample of the L-A group. This phenomenon could have a relation with a high relative abundance of *Bacillus*, the production of polyphenol oxidase by *Bacillus* plays a significant role in the catabolism of phenolic compounds (Mohammad & Alireza, 2007), ultimately leading to TPC degradation. The TFC in the C group ranged from 40.33 to 150.90 mg/100 g *Cupei*, as shown in Fig. 4B. The TFC in the L group, A group, L + A group, and L-A group were 157.78, 173.66, 127.23, and 113.21 mg/100 g *Cupei* at the end of fermentation, respectively. The highest TFC was observed in the A group, which was enhanced by approximately 15.08 % compared with the C group (Fig. 4B).

As shown in Fig. 5, a total of five organic acids were identified in each process. The acetic acid content rapidly increased, while the content of lactic acid slightly increased and then decreased in five groups during fermentation. The acetic acid was the highest with contents at the end of fermentation of five groups. Moreover, the highest acetic acid content was detected in the sample of the A group, and the highest lactic acid content was observed in the sample of the L group. The highest critic acid and malic acid contents were observed in the C group at the end of fermentation, with the content of 0.029 and 0.051 g/100 g *Cupei*. Due to oxalic acid originating from the hydrolysis of raw materials, the content of oxalic acid in the five groups merely slightly fluctuated during fermentation.

3.4. Volatile flavor compounds

As shown in Fig. 6A and Table S1, 96 VFCs were detected by SPME-GC–MS in five groups at the end of fermentation, including 43 esters, 11 acids, 8 alcohols, 12 aldehydes, 7 ketones, 6 pyrazines, and 9 others. The kinds of VFCs in the five groups had no obvious difference. Due to acetic acid and ethanol having already been discussed, it has not been included here. The heatmap and Venn diagram of VFCs in *Cupei* of five groups are shown in Fig. 6A-B. The results indicated that the VFCs were affected by bioaugmentation strategies. As shown in Fig. 6C, the contents of esters, ketones, and pyrazines in the L-A group were significantly higher than in other groups. In addition, the contents of acids had no obvious significant difference between the L+A group and L-A group, and they were higher than those of the three groups. OPLS-DA was performed on the VFCs at the end of fermentation (including ethanol and acetic acid). The score plot and HCA showed that the sample of the L-A group had an obvious difference compared with other groups (Fig. 7A-B). These results also indicated that the flavor was enhanced by sequential bioaugmentation strategy. Additionally, the variable importance plot (VIP)

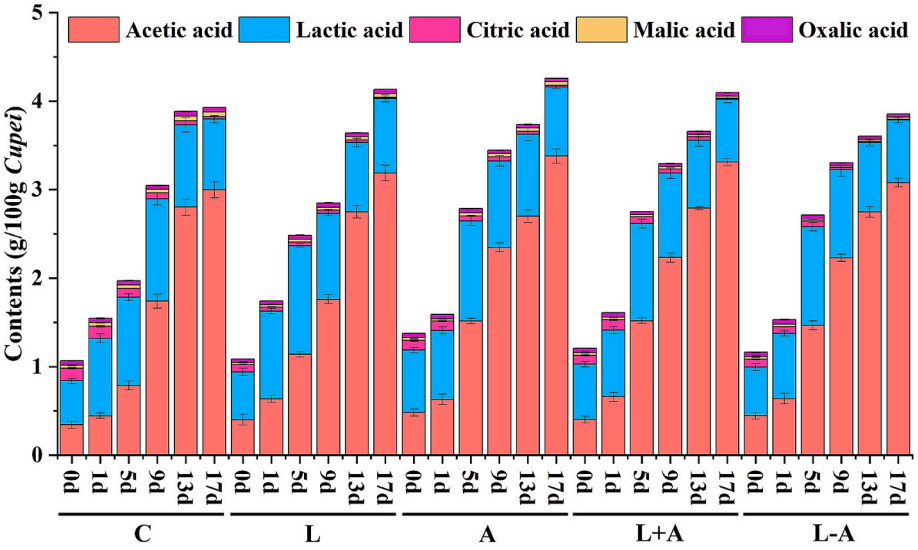

Fig. 5. Changes in organic acid contents during the fermentation.

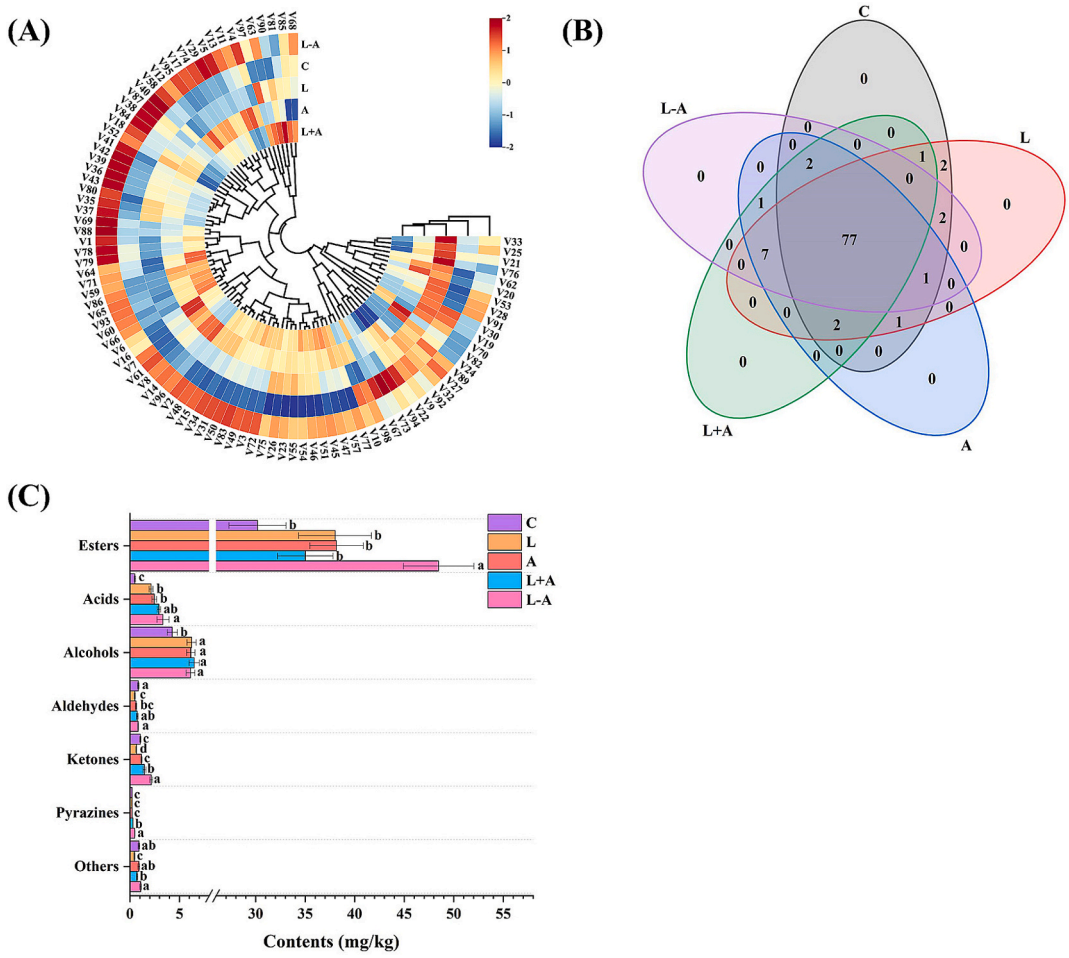

Fig. 6. The heatmap analysis (A), Venn diagram (B), and concentration (C) of VFCs in cereal vinegar fermentation bioaugmented by *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089.

was employed to highlight the significant variables (Fig. 7C). VIP values exceeding 1.0 for VFCs were considered significant effects on the bio-augmented fermentation, denoting them as differential VFCs. A total of 12 VFCs (acetic acid, ethanol, acetoin, 2,3-butanediol, ethyl 2-hydroxy-4-methylvalerate, phenylethyl alcohol, ethyl acetate, ethyl linoleate, ethyl palmitate, ethyl oleate, ethyl succinate, and 3-methyl-butanoic

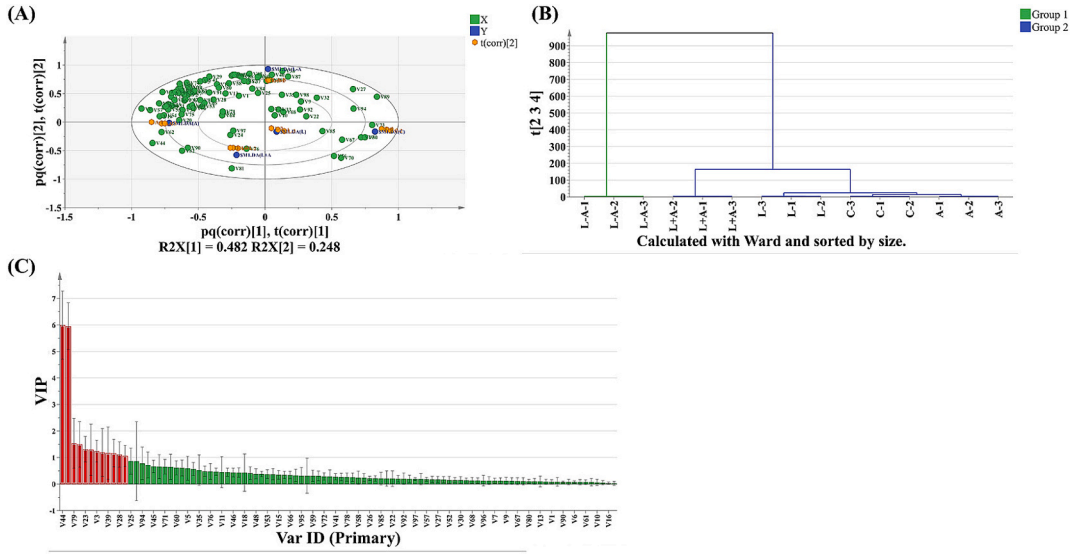

Fig. 7. OPLS-DA of the detected VFCs. The bioplot (A), HCA (B), and the VIP values (C).

acid) might perform as a vital indicator of cereal vinegar fermented by different bioaugmentation strategies.

3.5. Sensory evaluation

The results of sensory evaluation in different bioaugmentation strategies are shown in Fig. S2A. The color and posture scores in the C, L, A, L+A, and L-A groups have no obvious difference. The taste scores of the L+A group were the highest, and this result is consistent with that of TTA. The odor has no obvious difference in the C and L groups, while the odor score of the L-A groups was obviously higher than the C, L, A, and L+A groups. The relation between flavor and sensory scores was determined by PLS-DA. As shown in Fig. S2B, all samples were separated into four groups: C (Cluster 1); L (Cluster 2); L+A and A (Cluster 3); and L-A (Cluster 4). These sensory attributes were well related to some flavors, e. g. taste and acetic acid, odor and esters, pyrazines (Fig. S2B). These results indicated that sequential bioaugmentation strategies could have a vital role in improving the quality of cereal vinegar.

4. Discussion

The fermentation process of traditional fermented foods depends on the manual experience, and the challenges of inconsistent quality and extended fermentation times remain unresolved in industrial production. It is pressing to resolve these problems by bioaugmentation strategies. Due to LAB and AAB widely existing in vinegar, their role is crucial in enhancing the quality of the vinegar (Ndoye et al., 2006; Pereira et al., 2020). *L. helveticus* and *A. pasteurianus* were frequently used as starters in cereal vinegar production (Ye et al., 2023). In the present study, *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 were isolated from traditional cereal vinegar fermentation and were employed in SSF to perfect the fermentation process and quality of cereal vinegar. The content of ethanol increased in the C group at the early stages, which could have a relation with the hetero-lactic fermentation of LAB (hetero-lactic fermentation produces lactic acid with carbon dioxide, ethanol, or acetic acid as by-products) (Tomita et al., 2017). The L group improves the abundance of homo-lactic fermentation of LAB which produces lactic acid without carbon dioxide. Therefore, the utilization of raw materials and acid production efficiency were improved. Additionally, the AAF is considered to finish when the content of ethanol is below 0.8 g/100 g *Cupei* in the manufacturing enterprise. Therefore, the fermentation time of bioaugmented groups was obviously shortened compared with that of the C group. In addition, the highest organic acid content was observed in the sample of the A group (Fig. 4), hence, the highest sour taste could be observed in the A group. The non-volatile acid content was enhanced in the early stages, and the ethanol utilization was boosted at the middle and middle-late stages by a sequential bioaugmentation strategy. Moreover, the sequential bioaugmentation strategy can enhance the production intensity of AAF by improving the utilization ratio of starch raw materials due to low acetic acid inhibition in the early stage.

Firmicutes and Proteobacteria were the dominant phyla in cereal vinegar fermentation (Fig. 1A), and the results followed the previous study (Ye et al., 2023). In the C group, *Lactobacillus* was observed as the dominant genus at the middle and late stages of the C group, while *Limosilactobacillus* was the main genus at the early and middle-early stages (Fig. 1B). By bioaugmenting *L. helveticus* CGMCC 12062 or *A. pasteurianus* CGMCC 3089, the relative abundance of *Lactobacillus* and *Acetobacter* were enhanced, while the relative abundance of *Limosilactobacillus* was reduced (Fig. 1B). The reason for the decrease of *Limosilactobacillus* abundance might be that bacteriocin produced by *L. helveticus* CGMCC 12062 or acetic acid produced by *A. pasteurianus* CGMCC 3089 restrained the growth and propagation of *Limosilactobacillus* (Angelescu et al., 2022; Sun et al., 2023). Acetic acid exhibits strong toxicity on microorganisms (Sun et al., 2023), bacterial diversity could be reduced in the early stage by bioaugmentation of *A. pasteurianus* CGMCC 3089 due to high acetic inhibition. However, the bacterial diversity of SSF of cereal vinegar was enhanced by co-bioaugmentation or sequential bioaugmentation of *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089, especially sequential bioaugmentation strategy reduced the acetic acid inhibition in the early stage compared with that of A group. Therefore, sequential bioaugmentation is an effective strategy for reducing acetic acid inhibition and improving microbial diversity, and some other studies have used sequential bioaugmentation strategy to improve product flavor and quality (Rabelo et al., 2024). Furthermore, the bacterial diversity could be affected by temperature changes caused by different bioaugmentation strategies. The phenomenon was also observed in other fermented foods. e.g. shrimp paste (Yang et al., 2023). In addition, the highest bacterial diversity of cereal vinegar was observed in the L group, this phenomenon is consistent with the previous study (Zhang et al., 2021), meanwhile, the bacterial diversity of the L-A group was also higher than C group, and A group, and it had no obvious difference compared with L+A group. The lactic acid produced by *Lactobacillus* and acetic acid generated by *Acetobacter* were improved by sequential bioaugmentation, which can be applied as a substrate for esterification

(Mehta et al., 2020), therefore, it strengthened the vinegar flavor. Furthermore, the mono-bioaugmentation of *L. helveticus* CGMCC 12062 shortened alone the fermentation time by 2 days, whereas mono-bioaugmentation of *A. pasteurianus* CGMCC 3089 produced an amount of acetic acid to decline bacterial diversity. In our previous study, *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 have an ecological relation of amensalism (Xia et al., 2020), and the mutual regulatory mechanism of *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 are shown in Fig. S3. Therefore, the sequential bio-augmentation strategy was an effective means for improving bacterial diversity and lactic acid content in the early stage, concurrently enhancing ethanol utilization and shortening fermentation time in the middle and late stages.

The relationship between VFCs and bacteria in vinegar has been reported in previous studies (Erturkmen et al., 2024; Maske et al., 2024). The bioaugmented by *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 may influence the profiles of VFCs by altering the bacterial community structures in the SSF process. The difference of VFCs in five group samples at the end of fermentation was determined by OPLS-DA to further analyze the effect of bioaugmentation (Fig. 7A-C). Twelve differentiated VFCs were confirmed via VIP value, including 6 esters, 2 acids, 3 alcohols, and 1 ketone, and the details of VIP value are shown in Fig. 7C. Esters are a paramount class of flavor compounds in the realm of fermented foods, owing to their elevated volatility and their profound impact on human olfactory perception (Lee & Ahn, 2009). Ethyl acetate, a prevalent flavor component in cereal vinegar, can provide sweet, fruity, and floral flavors (Guneser et al., 2022). *L. helveticus* and *A. pasteurianus* had the ability to produce esterification enzymes (Kashima et al., 1998; Lo Verde et al., 2022), in this present study, the content of ethyl acetate in different bioaugmentation groups both were enhanced, and the highest ethyl acetate was observed in L-A group at an impressive 3.834 mg/kg *Cupei* (Table S1). This phenomenon could be due to the improvement of the metabolic activity of L. *helveticus* by minimizing acetic acid inhibition in the early stages, and *A. pasteurianus* plays a vital role in esterification enzyme formation at the late stage of fermentation. Furthermore, esterification enzymes also play a pivotal role in ethyl linoleate, ethyl palmitate, ethyl oleate, ethyl 2-hydroxy-4-methylvalerate, and ethyl succinate formation, which these flavor compounds impart the unique odor of vinegar. 3-methyl-butanoic acid has a sour odor and can produce unpleasant (Brunschwig et al., 2012). The highest concentration of 3-methylbutyric acid was observed in the L+A group, attributed to their combined effects on lipid oxidation, phospholipid hydrolysis, and triglyceride hydrolysis (Liao et al., 2024). Phenylethyl alcohol can provide sweet, floral, and cocoa-like flavor, and 2,3-butanediol and acetoin can provide a creamy, buttery. (Tian et al., 2023). The highest content of 2,3-butanediol and the lowest content of acetoin were observed in the L group (Table S1). *L. helveticus* converts sugars to pyruvate via the EMP pathway and further converts pyruvate to lactic acid or 2,3-butanediol (Tian et al., 2023). AAB can produce acetoin by acetolactate synthase (EC: 2.2.1.6) and dehydrogenation of 2,3-butanediol (Pelicaen et al., 2020; Zhou et al., 2018), and co-bioaugmented AAB and LAB can accelerate the accumulation of acetoin (Moens et al., 2014). In the present study, the highest content of acetoin was observed in the L-A group, with an amount of 1.903 mg/kg *Cupei* (Table S1). Bioaugmentation of *L. helveticus* could reduce the relative abundance of hetero-lactic fermentation LAB and improve the content of lactic acid, meanwhile, bioaugmented *A. pasteurianus* at the middle-early stages could produce acetoin by oxidation of lactic acid (Maske et al., 2024). Furthermore, TMP is also a vital flavor and functional component, leading to pleasant nutty and roasted vinegar (Ozdemir et al., 2022; Rios-Rein et al., 2020; Xu et al., 2018). TMP is mainly generated through the Maillard reaction due to an abundance of acetoin and sugar with nitrogen. The highest content of TMP was observed in the L-A group (0.373 mg/kg *Cupei*), the reason may be that the high acetoin and the high fermentation temperature accelerate the generation of the Maillard reaction (Basso et al., 2024). Therefore, enhancing acetoin by sequential bioaugmentation strategy during SSF is a feasible strategy to promote TMP accumulation.

5. Conclusion

Two autochthonous strains of *L. helveticus* CGMCC 12062 and *A. pasteurianus* CGMCC 3089 were used in SSF of cereal vinegar by different bioaugmentation strategies for targeted regulating the fermentation process and flavor of cereal vinegar. The highest bacterial diversity was observed in the L group, while the bacterial diversity of the sequential bioaugmentation strategy could be improved by minimizing early-stage acetic acid inhibition compared to the A group bio-augmentation strategies. Furthermore, the sequential bioaugmentation strategy enhanced non-volatile acid content by reducing early-stage acetic acid inhibition and subsequently boosted ethanol conversion efficiency by inoculating *A. pasteurianus* CGMCC 3089. Therefore, the utilization ratio of starch raw materials and fermentation efficiency were significantly enhanced by the sequential bioaugmentation strategy. Besides, the sequential bioaugmentation strategy targetedly improved the acetoin and TMP contents. These results may promote the understanding of selective starters on the effect of cereal vinegar fermentation, and targeted regulate the fermentation and flavor by sequential bioaugmentation strategy. However, this study was conducted in winter, and traditional cereal vinegar fermentation is known to be influenced by seasonal variations. Consequently, the impact of a sequential bioaugmentation strategy on cereal vinegar fermentation warrants further validation across different seasons in future research.

Ethical statement

The ethical permission of the sensory studies of Sequential Bioaugmentation of the Dominant Microorganisms to Improve the Fermentation and Flavor of Cereal Vinegar in the Entire Production Process was granted by Ethics Committee of Tianjin University of Science and Technology. All participants have read introduction about this research, known the possible risks and benefits of participating in this research, agreed that the ethics Committee of the supervision and management department, got a signed and dated copy of the informed consent form, and decided to agree to participate in this research.

CRediT authorship contribution statement

Ao Zhang: Writing – original draft, Visualization, Investigation, Formal analysis. **Wenqing Zhang:** Software, Formal analysis, Data curation. **Xiaorui Guo:** Software, Investigation. **Jiao Wang:** Resources, Investigation, Data curation. **Kai Liang:** Validation, Investigation, Conceptualization. **Yaao Zhou:** Visualization, Investigation, Data curation. **Fanfan Lang:** Software, Resources, Formal analysis. **Yu Zheng:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition. **Min Wang:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors have declared no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (32472321), Innovation Fund of Haihe Laboratory

of Synthetic Biology (22HHSWSS00013), Key Research and Development Projects of Shanxi Province (202202140601018), Shanxi Provincial Department of Science and Technology (202204010931002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101952.

Data availability

No data was used for the research described in the article.

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