



Apple spontaneous fermentation sourdough: Microbial dynamics and optimization for enhanced bread quality

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Abstract: This study explored using apple-derived spontaneous fermentation sourdough to replace commercial cultures in bread production, analyzing its microbial composition and optimizing process parameters. Microbial profiling via 16S and 26S rDNA sequencing identified *Lactobacillus plantarum* as the dominant lactic acid bacteria. SFS addition levels (0–30%) and proofing times (60–180 minutes) were varied to assess impacts on bread quality. Results revealed that shorter proofing initially increased specific volume, which stabilized over time, while hardness decreased and then increased. Similarly, lower apple-derived spontaneous fermentation sourdough percentages enhanced volume, but higher doses reduced it, with hardness showing a comparable trend. Lower apple-derived spontaneous fermentation sourdough has improved nutritional properties (free amino acids) and flavor (volatile compounds). The findings demonstrate lower apple-derived spontaneous fermentation sourdough's potential to enhance bread quality, offering natural alternatives to commercial starters by balancing fermentation parameters to optimize texture, volume, shelf-life, and sensory attributes, highlighting its role in sustainable, artisanal bread production.

Keywords: spontaneous fermentation sourdough; microbial community structure; isolation and identification; baking properties; volatile flavor compounds

1. Introduction

Naturally fermented sourdough is a natural mixed starter obtained by natural fermentation of grains, fruits, and vegetables or other substrates with water and active microorganisms (mainly lactic acid bacteria and yeast flora). The expression "natural yeast" is often used in the baking industry, and naturally fermented sourdough is a typical natural yeast system ([Islam & Islam, 2024](#)). It is one of the oldest biological technologies in China and even in the world to ferment dough products by using naturally fermented sourdough as a starter. As early as the Zhou Dynasty, Chinese people mastered the technology of making steamed bread using "wine noodles", where "wine noodles" are the earliest recorded naturally fermented sour dough; during the Song Dynasty (AD960 ~ 1279), the "fermented flour fermentation method" appeared to produce steamed bread. "Fermented flour" continues to develop into the main form of naturally fermented sourdough in China at present ([Wang et al., 2018](#)).

In Western countries, however, the natural sourdough technology is mainly used in bread. Foreign technology of naturally fermented sourdough bread began in Egypt in 1500 BC and has a history of more than 5000 years. With the development of social civilization, people in North America and central Europe gradually mastered the use of natural fermentation techniques to produce wheat, rye bread, etc ([Lau et al., 2021](#)). Today, 30% of bread production in Italy is still done by natural fermentation on sourdough. Up to now, natural sourdough technology still exists firmly in the bread-making industry in Western countries, with its unique advantages, and has been studied extensively ([Hernández-Figueroa et al., 2023](#)). With the rise and popularity of bread products in China, domestic research on the production of bread using natural fermentation sourdough technology has also begun. Since its invention in the 1860s, commercial yeast has been rapidly popularized in the bakery industry due to its long storage time, quick and easy use, and strong controllability ([Akamine et al., 2023](#)). But at the same time, the common commercial yeast bread became the shackles of its development because of its single flavor, rough taste, and heavy yeast flavor. The diversified demand of consumers for food quality has brought back the natural sourdough technology that endows bread products with natural health,

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rich nutrition, and good quality (Chavan & Chavan, 2011; Islam & Islam, 2024). Raisins are often used as the best source of cellulose and natural sugar added to bread to enhance the nutritional value and taste of bread products. There are a few reports on the application of raisin fermentation products in food (Abdollahzadeh et al., 2024). Apple is the main fruit consumed in many countries in the world, which has the advantages of strong ecological adaptability, high nutritional value, and storage resistance.

In addition to being rich in vitamins and minerals, apples are an important dietary source of phenols and have considerable antioxidant properties (Arnold & Gramza-Michalowska, 2024). Apples are mainly used in food production to produce apple juice. Apple fermentation products are mainly used in the production of apple drinks, apple vinegar, and apple cider, and less in bread (Tsoupras et al., 2024). Gray and Bemiller (2003) used fermented apples for bread production to improve the gas-holding properties of bread. Fermentation substrate has a great influence on the quantity of yeast, but a small influence on the species, mainly *Saccharomyces cerevisiae*; different fermentation substrates have a small influence on the quantity of lactic acid bacteria, but a large influence on the species.

This study (1) aimed develop production methods for apple-based spontaneous sourdoughs by monitoring sugar/acid metabolism to determine optimal fermentation endpoints; (2) isolate and characterize lactic acid bacteria to clarify their roles in enhancing bread quality; (3) evaluate the superior baking performance and nutritional benefits (e.g., free amino acids) of sourdough bread compared to commercial yeast bread; and (4) analyze flavor enhancement and storage advantages imparted by spontaneous sourdough, elucidating mechanisms behind delayed staling and improved sensory properties. The work underscores sourdough's potential as a natural, multifunctional alternative to industrial starters.

2. Materials and Methods

2.1. Experimental Materials and Equipment

2.1.1. Experimental materials and reagents

Wheat Flour: COFCO Flour Industry Pengtai Co., Ltd.; Instant Dry Yeast: Guangdong Meishan Mali Yeast Co., Ltd.; Shortening: Donghai Grain and Oil Industry (Zhangjiagang) Co., Ltd.; Sugar, Salt, Apple, Honey: Provided by Fujian Anmai Biotechnology Co., Ltd.

Glucose, fructose, maltose, lactose, sucrose, lactic acid, acetic acid, perchloric acid, sodium hydroxide, sodium chloride, peptone, beef extract, yeast extract, potassium hydrogen sulfate, anhydrous sodium acetate, ammonium citrate, MgSO₄·7H₂O, MnSO₄·4H₂O, Tween 80, agar powder: Sinopharm Chemical Reagent Co., Ltd.; actinomycin: Shanghai Baoman Biotechnology Co., Ltd.; bacterial genome DNA extraction kit, yeast genome DNA extraction kit: Tiangen Biochemical Technology (Beijing) Co., Ltd.; primers and enzyme preparations: Shanghai Sunny Technology Co., Ltd. Lactic acid bacteria culture medium (MRS): yeast extract 5g, beef extract 10g, glucose 20g, peptone 10g, Ammonium citrate 2 g, anhydrous sodium acetate 5 g, dipotassium hydrogen sulfate 2 g, MgSO₄·7H₂O 0.58 g, MnSO₄·4H₂O 0.25 g, 1 mL Tween 80, 1000 mL distilled water, pH 6.2-6.6, sterilized at 115 °C for 20 min, and 20g agar powder was added to the solid medium.

2.1.2. Main equipment

JY20002 electronic balance: Shanghai Liangping Instrument Co., Ltd.; mixer, incubator, electric oven, microtome: Xinmai Machinery Co., Ltd.; CT3 texture analyzer: Brookfield Company; constant temperature and humidity incubator: Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory; FE20 laboratory pH meter: Switzerland Toledo Company; Waters 244 high performance liquid chromatograph: Agilent Technology; XSP-10 electron microscope: Shanghai Biaimu Optical Instrument Manufacturing Co., Ltd.; SHY-2A water bath constant temperature oscillator: Jiangsu Jincheng Guosheng Experimental Instrument Factory; DYY-8C electrophoresis instrument: Beijing City Liuyi Instrument Factory; 5427R desktop high speed refrigeration centrifuge, Mastercycler nexus X1 PCR instrument, Eppendorf BioPhotometer D30 nucleic acid protein analyzer: Eppendorf (China) Co., Ltd.

2.2. Experimental method

2.2.1. Preparation of naturally fermented sourdough

The selection of fruit substrate in naturally fermented sourdough follows the following principles: a wide source and easy storage; high sugar content suitable for microbial growth; low seasonality, and the season has



little effect on the final quality of the product. In the preliminary experiment, apple was chosen as a fermentation substrate, bubbles were generated evenly, and repeatability was good, so it was finally selected as a fermentation substrate to prepare naturally fermented sourdough.

Weigh 100g apples, wash them with warm boiled water, cut them into pieces and pour them into sterilized wide-mouth glass bottles, add 10g honey and 200 g sterilized distilled water cooled to 30°C respectively, seal them with cotton plugs, and put the glass bottles into constant temperature of 30°C in a wet box, culture for 5-7 days to obtain stable product, and filtering with sterile gauze to obtain fermentation broth.

2.3. Determination of pH of the fermentation broth

During the natural fermentation of apples until the fermentation broth in 2.2.1 is obtained, the pH value of the fermentation broth on d 0, d 1, d 3, d 5, and d 7 is directly measured by a pH meter.

2.3.1. Determination of soluble sugar in fermentation broth

Soluble sugar content of fermentation broth was determined at 0, 1, 3, 5 and 7 days. According to the extraction method of Zhang Qing, dilute the solution to 100 mL, centrifuge at 4000 r/min for 10 min, and collect the supernatant. The supernatant was assayed by HPLC (High Performance Liquid Chromatography). Among them, glucose, fructose, lactose, maltose, and sucrose were used as standard samples.

2.3.2. Determination of Organic Acids in Fermentation Broth

The organic acid content of the fermentation broth was determined. Add 5 mL of 1 mol/L HClO₄ to 10 mL of fermentation broth, mix well, centrifuge at 5000 r/min for 15 min at 4°C, add distilled water to make 25 mL of supernatant, precipitate in an ice bath, microfilter the supernatant (0.45 μm), and then perform HPLC analysis. Determine the contents of lactic acid and acetic acid in the fermentation broth of the sample with lactic acid and acetic acid as standard samples.

2.3.3. Determination of basic properties of naturally fermented sourdough

2.3.3.1. Sample preparation

Take the naturally fermented sourdough finally obtained.

2.3.3.2. Determination of pH and total acidity of naturally fermented sourdough

The pH and total acidity of naturally fermented sourdough from apples were determined.

According to the AACC method (2000) 02-52, respectively, take 10 g of sample into a conical flask, add 90 mL of distilled water, magnetically stir for 30 min, stand for 10 min, and determine pH. Titrate with 0.1 mol/L NaOH and stir to pH 8.6; the volume of NaOH consumed is the total acidity. Repeat the operation at least three times for each sample.

2.4. Counting of lactic acid bacteria in naturally fermented sourdough

Ten (10) g of apple sourdough was mixed with 90 mL at a concentration of 0.85%. The sterile saline solution was mixed and diluted tenfold ([Leng, 2011](#)). Lactic acid bacteria count: MRS solid plate coating method. Take seven dilution gradients of 10⁻¹-10⁻⁷, apply 100 μL, incubate at 38°C for 48 h, and count.

2.5. Identification of lactic acid bacteria and yeasts in naturally fermented sourdough

2.5.1. Isolation, purification, and preservation of lactic acid bacteria from naturally fermented sourdough

a. Preparation of bacterial suspension

Mix 10 g of apple sourdough with 90 mL of sterile saline, shake well. Dilute to 10⁻⁸ in steps.

b. Isolation, purification, and preservation of lactic acid bacteria

The isolation, purification, and preservation steps of lactic acid bacteria are shown in Figure 1.

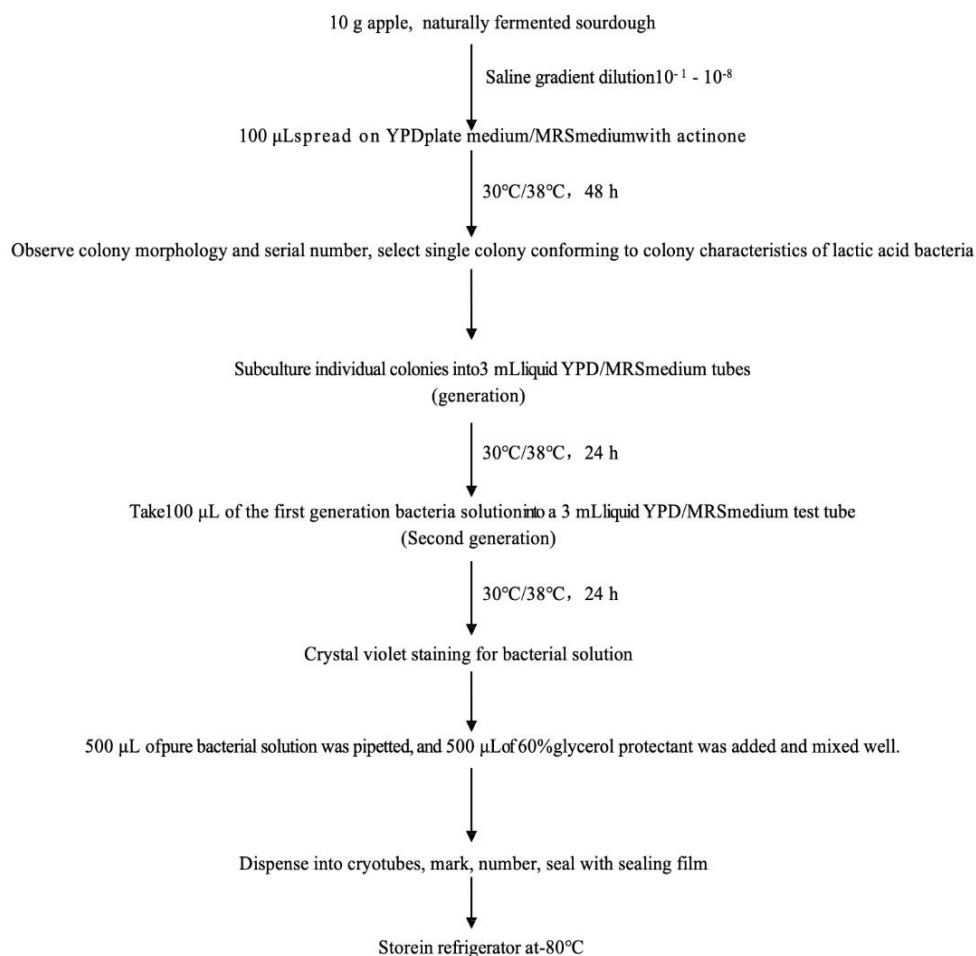


Fig.1: Procedure for isolation, purification, and conservation of strains

2.6. Identification of lactic acid bacteria isolates from naturally fermented sourdough

a. DNA Extraction and Purity Test of Isolated Strains

DNA of isolated strains was extracted using the bacterial genomic DNA extraction kit and the yeast genomic DNA extraction kit. Take 1 μL of yeast and lactic acid bacteria DNA stock solution, and detect the OD ratio and concentration of DNA extracted by a micro-ultraviolet spectrophotometer. After sequencing of amplified products, sequence splicing was carried out by DNASTAR software, and the obtained base sequence was compared with the NCBI database for Blasthomology, and the sequence of known species with the closest homology was found (Leng, 2011).

2.7. Preparation of naturally fermented sourdough bread

Naturally fermented sourdough bread has slow microbial action, so the method of secondary fermentation is used to make bread. Process: Mix seed dough components, pre-ferment at 30 $^{\circ}\text{C}$ for 4.5h, add dry ingredients and water, and stir slowly, add shortening and stir quickly for 3 min until a gluten network structure is formed, stirring slowly for 1 min, coating and standing for 10 min at room temperature.

Process: dry materials mixed evenly, add water slowly, stir into dough, add shortening and stir quickly for 3 min until a gluten network structure is formed, stir slowly for 1min, cover and stand for 10 min at room temperature. Divide the dough into 90 g pieces, and roll them into round shapes. Naturally fermented sourdough bread was proofed for 4 h, 6 h, and 8 h, respectively, in a proofer (30 $^{\circ}\text{C}$, relative humidity 85%). Common yeast bread was proofed for 90 min in a proofer (38 $^{\circ}\text{C}$, relative humidity 85%). The proofed dough was put into the oven (170 $^{\circ}\text{C}$, 210 $^{\circ}\text{C}$) and baked for 25 min.



2.7.1. Determination of baking characteristics of naturally fermented sourdough bread

2.7.2. Determination of the specific volume of bread

After the bread is cooled for 2h, the volume is determined by the rapeseed displacement method (Liu, 2014), and the mass and specific volume are determined by an electronic balance (mL/g).

2.7.3. Determination of bread hardness

The hardness of the cooled bread was measured by a texture analyzer with different additive amounts and different proofing times. Cut the bread into uniform slices with a thickness of 12.0 mm, and take the middle two slices to measure the hardness of the bread core. Parameter settings: test type TPA, probe model P/36, induction force 5g, pre-test rate 1.0mm/s, test rate 3.0mm/s. The post-test velocity is 3.0 mm/s, the compression degree is 50%, and the interval between two compressions is 1s.

2.7.4. Analysis of free amino acids in naturally fermented sourdough bread

Free amino acids of two kinds of sourdough bread (apple) fermented naturally by the best technology were determined and analyzed, and common yeast bread was used as a control. Take 1 g of dried bread sample, add 15mL trichloroacetic acid, 10000 Centrifuge at r/min for 10min, filter with filter paper, transfer supernatant to a 25 mL volumetric flask, centrifuge at 10000r/min for 10min after constant volume, and analyze free amino acids by HPLC after pre-column derivatization of o-phthalaldehyde. ODS Hypersil column (250mm×4.6 mm×5μm) was used. The column temperature was 40°C. The mobile phase was 20 mmol/L sodium acetate and 1:2 (vol/volume) of methanol-acetonitrile at a flow rate of 1mL/min (Zhang Wei, 2014).

2.7.5. Determination of volatile flavor compounds in naturally fermented sourdough bread

Volatile flavor compounds of two kinds of sourdough bread (apple) fermented naturally by the best technology were determined and analyzed, and common yeast bread was used as a control.

2.7.6. Headspace Solid Phase Microextraction (SPME) of Volatile Flavor Components

Divide the bread into pieces of 5 mm×5 mm×3 mm, place them in a 15 mL SPME sample bottle, and then insert the extraction head from the top of the sample bottle into a constant temperature water bath at 60°C. After headspace extraction for 40 min, inject the sample.

2.8. GC-MS analysis

Chromatographic conditions: DB-5MS capillary column (60 m×0.32 mm, 1 μm) chromatographic column; carrier gas is He; heating program: constant 40°C, 1min after 6°C/min to 160°C, then 10°C/min to 250°C, keep 10min; 1.2mL/min constant flow for 2 min, then split at a rate of 10 mL/min and a split ratio of 12:1. Mass spectrum conditions: electron ionization, injection hole temperature 250°C, electron energy 70eV, ion source temperature 200°C, emission current 200 μA. The acquisition mode is full scan, and the acquisition mass range is m/z 33-495.

2.8.1. Qualitative and quantitative analysis of volatile flavor compounds

GC-MS peaks were matched with the Wiley Library and the NIST Library by computer and manual retrieval, and the identification results were set as matching degree and purity greater than 800. Compound quantification: Calculate the relative percentage content according to the peak area normalization method.

2.9. Analysis of storage characteristics of naturally fermented sourdough bread

2.9.1. Determination of texture parameters of bread during storage

The bread was sealed and stored at 4°C. The texture parameters of bread stored at 0, 1, 3, 5 and 7 days were determined by a texture analyzer.

2.9.2. Determination of moisture migration during bread storage

The bread was sealed and stored at 4°C for accelerated aging. The retrogradation enthalpy of amylopectin was determined by DSC at 0, 1, 3, 5, and 7 days after storage. Weigh 10mg of sample, temperature rise rate is 5°C/min, scan range is 30-110°C (Zhang Wei, 2014).

2.10. Data analysis and processing

Excel 2007 and SPSS 19.0 software were used for statistical analysis of the data, and analysis of variance (ANOVA) was used to analyze significance at an $\alpha < 0.05$ level. DesignExpert 8.0 was used to design and analyze response surface experiments.

3. Results and discussion

3.1. Metabolization of substances in fermentation broth during fermentation

The preparation of the apple fermentation broth is the main step in making two kinds of naturally fermented sourdough. During the incubation period, acid flavor and ethanol smell appear in the fermentation broth, accompanied by bubble formation. In order to determine the endpoint of fermentation, this part explored the metabolic behavior of microorganisms in fermentation broth.

3.2. pH change of fermentation broth

It was observed that at 0d, the initial pH value had little difference, and with the increase of fermentation time, the pH value decreased to different degrees, and the decrease was most obvious at 1-3d. At the later stage of fermentation (5-7d), the pH value of fermentation broth remained basically unchanged due to high acidity inhibiting the growth of microorganisms. Lactic acid bacteria brought in by fruits mainly exist in the natural fermentation liquid of fruits. Lactic acid bacteria usually carry out metabolic reactions in an anaerobic or facultative anaerobic manner. In the process of fermentation, organic acids such as acetic acid, lactic acid, and propionic acid are produced, imparting acidic flavor to the fermentation broth, and at the same time, various acids produced inevitably cause the pH of the fermentation broth to drop. The type, activity, and quantity of lactic acid bacteria have different effects on the pH value of fermentation broth (Ma Xia et al., 2013), and the reason for the significant difference in pH value of the two fermentation broths may be due to the different initial raw materials selected (apples), resulting in different lactic acid bacteria generated during natural fermentation. Yeast had little effect on the pH of the fermentation broth.

3.3. Changes of Organic Acids in Fermentation Broth

The main microorganisms in the apple fermentation broth were lactic acid bacteria and yeast. Lactic acid bacteria can pass through EMP and HMP, two metabolic pathways (Hidayati et al., 2024), and their main products are lactic acid and acetic acid. Therefore, two organic acids, lactic acid and acetic acid, are selected as standard samples for the determination of organic acids in two fermentation solutions in this study. The concentration of organic acids in samples is determined by comparing the concentration of standard samples with high-performance liquid chromatography (HPLC).

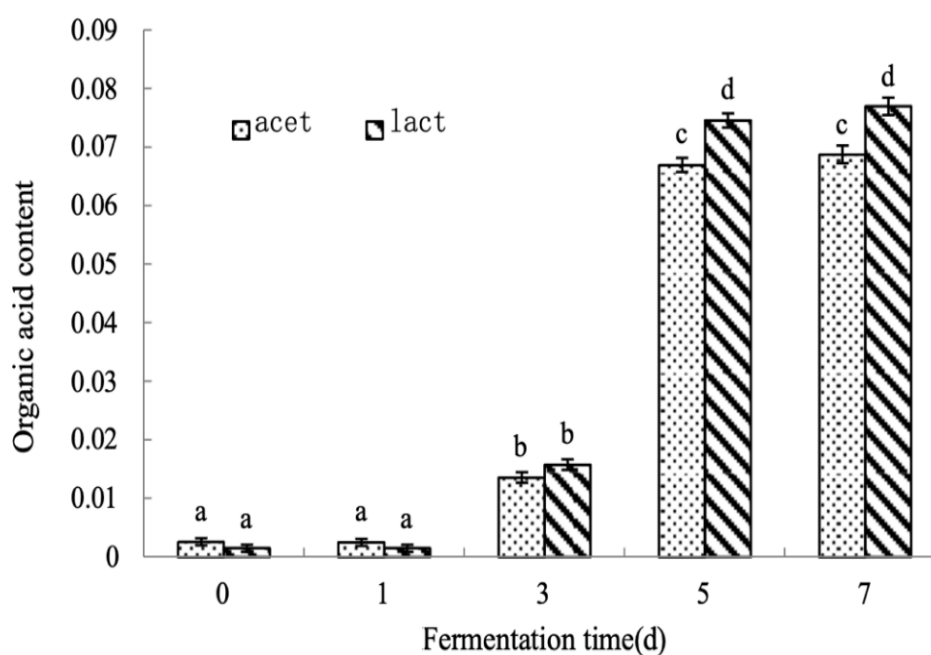


Fig. 2: The changes in organic acid content in apple broth during the fermentation process



As can be seen from Figures 1 and 2, in the two fermentation solutions, at 0d, natural fermentation has not yet started, and the acetic acid and lactic acid contents in the fermentation solution are extremely low, which may be introduced by apples. With the fermentation time increasing, lactic acid bacteria fermentation acid production, the content of two kinds of organic acids in the fermentation broth gradually increased, the organic acid increased to the maximum within 3-5 days, and within 5-7 days, with the consumption of carbohydrates completely, the organic acid content increased slowly, basically unchanged.

According to the experimental results of pH, soluble sugar and organic acid, the pH of fermentation broth remained basically unchanged at 7d, the main soluble sugar was consumed, and the content of organic acid did not increase, the metabolic activities in the fermentation broth had reached a relatively stable state, indicating that the microorganisms in the fermentation broth were also in dynamic equilibrium, so 7 d was selected as the fermentation endpoint of apple fermentation broth. After fermentation for 7 days, the fermented liquid is added to high-gluten flour and cultured for 5-6 hours to obtain naturally fermented sourdough.

3.4. Basic properties of naturally fermented sourdough

The value of TTA refers to the total acid content of naturally leavened sourdough, while pH indicates the amount of strong acid produced by microorganisms in naturally leavened sourdough. Table 3.1 shows the pH and TA values and the number of lactic acid bacteria and yeast contained in apple sourdough. From Table 3.1, it can be seen that the pH values of the two naturally fermented sourdoughs are 5.08 and 3.88, respectively, and the corresponding TTA is 7.70 mL and 12.32 mL. The pH of the naturally fermented acid dough of apples is lower, the acidity is stronger, and the corresponding total acid content is also higher.

The pH and TTA results are close to those of traditional sourdough in some regions of China. The study of Zhang showed that pH and TA of naturally fermented sourdough collected from western Inner Mongolia were limited to a narrow range of 3.32 - 3.82 and 11.2 - 12.9 mL, respectively. The main reason for the differences between naturally fermented sourdough is the number and type of microorganisms in naturally fermented sourdough in different regions, which will produce differences in acid production capacity.

Table 3.1: The pH, TTA, and result of counting lactic acid bacteria and yeast in two kinds of spontaneous fermentation sourdough

	pH value	TTA (mL)	LAB (log CFU/g)	Yeast (log CFU/g)
Apple naturally fermented sourdough	3.88±0.04	12.32±0.23	7.92±0.13	5.84±0.17

The counts of lactic acid bacteria and yeasts in Table 3.1 show that the average number of lactic acid bacteria in apple sourdough was 7.92 log CFU/g. According to literature reports, when the ratio of lactic acid bacteria to yeast in the naturally fermented sourdough system is 100:1, the microorganisms in the system reach a relatively stable state of existence.

The colony counts results in this study showed that the ratio of lactic acid bacteria in apple sourdough was close to 100:1, that is, both reached a stable state. More lactic acid bacteria in apple sourdough corresponded to lower pH values. Compared with other authors who counted $10^8 - 10^9$ CFU/g of lactic acid bacteria and $10^6 - 10^7$ CFU/g of yeast for naturally fermented sourdough, the numbers of lactic acid bacteria and yeast in this study were lower, probably due to the introduction of fruit substrate.

3.5. Isolation and identification of lactic acid bacteria from naturally fermented sourdough

3.5.1. Isolation and identification of lactic acid bacteria from naturally fermented sourdough

MRS solid medium was used to isolate lactic acid bacteria in this study. The colonies of lactic acid bacteria are usually white, smooth, and moist, with flat edges, colony diameters of 0.5-1.5 mm, and Gram-positive by microscopic examination. In this study, 12 strains were isolated from apple sourdough.

Table 3.2: The identification result of LAB based on 16S rDNA sequencing

strain number	identification results	homology
AL1	<i>Lactobacillus plantarum</i>	99%
AL2	<i>Lactobacillus plantarum</i>	100%
AL3	<i>Lactobacillus plantarum</i>	100%
AL4	<i>Lactobacillus plantarum</i>	100%
AL5	<i>Lactobacillus plantarum</i>	100%
AL6	<i>Lactobacillus plantarum</i>	100%
AL7	<i>Lactobacillus plantarum</i>	100%
AL8	<i>Lactobacillus plantarum</i>	100%
AL9	<i>Lactobacillus plantarum</i>	100%
AL10	<i>Lactobacillus plantarum</i>	99%
AL11	<i>Lactobacillus plantarum</i>	100%
AL12	<i>Lactobacillus plantarum</i>	100%

The strain could be identified as a species if the homology exceeded 99%. The identification results of lactic acid bacteria 16S rDNA in two kinds of naturally fermented sourdough are shown in Table 3.2. All of the above strains have 99% or 100% homology, so they can be identified as species. All strains were identified as *Lactobacillus plantarum*. It is also a facultative heterofermentative lactic acid bacterium with strong acid-producing ability. Its abundant existence also explains the low pH value of the naturally fermented sourdough.

3.6. Effect of naturally fermented sourdough on the baking characteristics of bread

Two technological parameters of sourdough addition and fermentation time of apple sourdough bread were selected and determined as 10%, 20%, 30%, 40%, and 4, 6, 8h, respectively.

3.6.1. Effect of naturally fermented sourdough on bread specific volume

The results are shown in Figure 3-8. Different combinations of natural sourdough addition and proofing time have different effects on the specific volume of bread. The bread specific volume increased firstly and then remained constant with the increase of proofing time when the additive content was constant, and increased firstly and then decreased with the increase of additive content when the proofing time was constant.

The content of yeasts in the naturally fermented sourdough system determines the ability of bread to produce gas, while lactic acid bacteria in the naturally fermented sourdough system can play an acidification role, and the type and degree of acidification will also have different effects on bread specific volume. Therefore, different combinations of these two important process parameters will have different effects on bread specific volume. The difference in specific volume between 4 h and 6 h is larger, but the difference between 6h and 8h is smaller. When the additive content was 40%, the specific volume decreased, probably due to excessive acidification. Under moderate acidity conditions, the specific volume of bread can reach a maximum.

The proper amount of acid will activate the egg white enzyme in wheat flour, jointly degrade gluten to release water, improve the water absorption rate of starch and polysaccharide, the activity of other enzymes, and the gas holding capacity of gluten network structure, while too high acidity and too strong protein hydrolysis will weaken gluten and reduce bread specific volume. At the same time, the symbiosis between lactic acid bacteria and yeast will increase the gas production of the yeast. There was a positive correlation between bread-specific volume and bread quality.

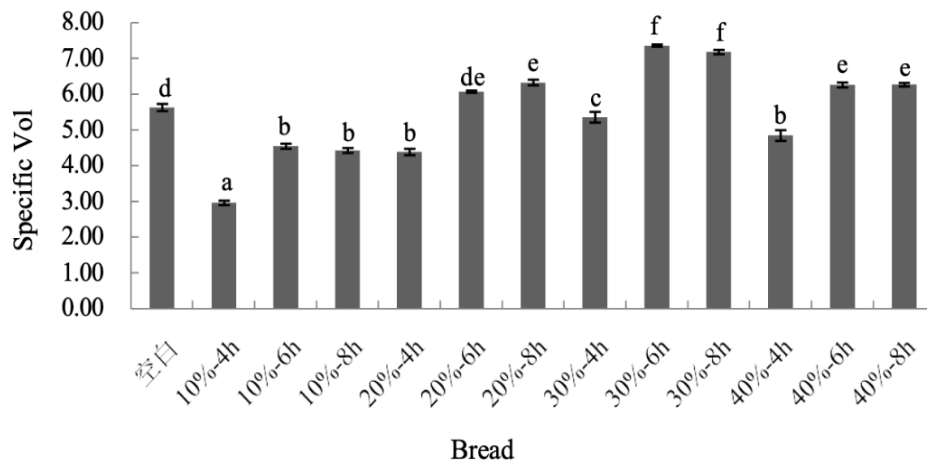
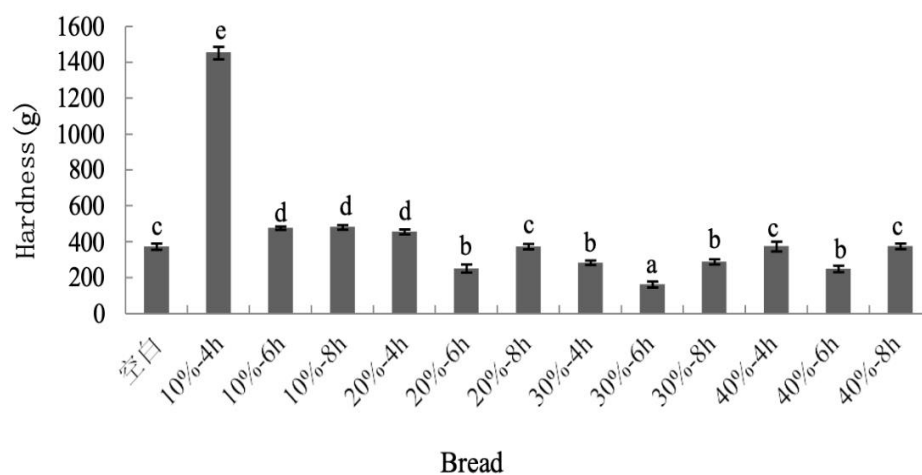


Fig. 3: Specific volume of different breads: Apple spontaneous fermentation sourdough bread

3.7. Effect of naturally fermented sourdough on bread hardness

Common yeast bread was used as the blank control group. The experimental results are shown in Figure 3. Hardness is one of the most important textural parameters to characterize bread quality. Different combinations of naturally leavened sourdough addition and proofing time will affect the hardness of bread. The hardness of the two kinds of bread reached its maximum at 10%-4h, which was due to the small amount of yeast added, the short time of bread proofing, the insufficient gas production of the dough, and the incomplete proofing, resulting in dense bread tissue and high hardness. With the increase in the amount of yeast added and the proofing time, the hardness of bread decreased to different degrees. The hardness of bread decreased firstly and then increased with the time of proofing when the amount of bread added was fixed, and decreased firstly and then increased with the amount of bread added when the time of proofing was fixed.

There was a negative correlation between bread hardness and bread quality. The above experimental results show that proper addition amount and proofing time are beneficial to reduce bread hardness and improve bread quality, while excessive addition amount leads to large acid production of lactic acid bacteria, excessive acidification of bread dough, resulting in increased bread hardness, which is consistent with Zhang Qing's research results. As shown in Figure 3-9, for grapes For dry sourdough bread and apple sourdough bread, the hardness minimum appeared in the 30%-6h group.



3.8. Effect of naturally fermented sourdough on nutritional properties of bread- free amino acids

Amino acids not only affect the flavor and color of bread, but also, as the basic component unit of protein, amino acids have nutritional support, immune regulation, anti-tumor, and other nutritional effects. Both naturally fermented sourdough breads contained 18 amino acids and tryptophan, which was not present in regular yeast bread. Tryptophan is an essential amino acid that can only be synthesized by plants or microorganisms. Tryptophan can promote sleep, relieve migraine headaches, and relieve anxiety and stress. The contents of 11 kinds of amino acids and 6 kinds of essential amino acids in bread were increased after adding naturally fermented sourdough from apples. After fermentation, the total amino acids increased, and the proportion of essential amino acids in total amino acids increased from 14.15% to 35.02% and 28.79%, respectively.

The increase in free amino acid content in naturally fermented sourdough bread is mainly due to protein degradation. *Lactobacillus plantarum* and *Lactobacillus sake* in the naturally fermented sour dough system ferment to produce acid, which stimulates the protease activity in flour, and the protease hydrolyzes protein, thus releasing free amino acids and small peptides, resulting in an increase in free amino acid content. Since yeast fermentation reduces the free amino acid content, we can assume that the use of naturally fermented sourdough instead of yeast fermentation can strengthen the amino acids in bread and make it more nutritious.

Compounds	Plain Yeast Bread	Apple Naturally fermented bread
glutamic acid	3.43533×10 ⁻²	2.28895×10 ⁻²
aspartic acid	5.52572×10 ⁻²	4.42513×10 ⁻²
serine	7.29053×10 ⁻⁴	3.60440×10 ⁻⁴
histidine	1.45920×10 ⁻³	1.41331×10 ⁻³
glycine	7.16986×10 ⁻³	8.40532×10 ⁻³
threonine	4.76751×10 ⁻³	6.33333×10 ⁻³
arginine	8.87450×10 ⁻³	1.03350×10 ⁻²
alanine	1.66298×10 ⁻²	1.46599×10 ⁻²
tyrosine	1.95145×10 ⁻³	3.02735×10 ⁻³
cysteine	2.88976×10 ⁻⁵	6.59661×10 ⁻⁵
valine	6.44574×10 ⁻³	9.86554×10 ⁻³
methionine	9.24600×10 ⁻⁴	6.41383×10 ⁻⁴
tryptophan	-	1.35858×10 ⁻²
leucine	2.24189×10 ⁻³	7.11628×10 ⁻³
lysine	4.23283×10 ⁻³	1.84217×10 ⁻³
proline	1.75837×10 ⁻²	2.04498×10 ⁻²

3.9. Characteristics of Volatile Flavor Compounds in Naturally Fermented Apple Sourdough Bread

The flavor of bread products is an important factor affecting bread acceptability. The metabolic activities of lactic acid bacteria and yeast coexisting in the naturally fermented sourdough system endow naturally fermented sourdough bread with special flavor characteristics. The metabolic products of yeast and lactic acid bacteria become the chemical components that determine this special flavor. The flavor substances produced by naturally fermented sourdough in bread are mainly divided into ketones, aldehydes, esters, and alcohols produced by biochemical action during fermentation and organic acids produced by lactic acid bacteria fermentation.



The GC-MS technique was used to determine and analyze the flavor compound characteristics of common yeast bread, naturally fermented raisin sourdough bread, and naturally fermented apple sourdough bread. The GC-MS spectra of flavor compounds of three kinds of bread are shown in Appendix A-4. After sourdough fermentation, the peak positions and peak areas in the spectra were different, and the types and relative contents of volatile flavor substances in bread were changed. From Table 3-5, we can see that 99% of the three kinds of bread were detected.

Volatile flavor compounds in the three breads were classified into eight categories as shown in Tables 3 - 6. Alcohol content is the highest, and natural fermentation sourdough makes its content further increase, among which ethanol, phenylethanol, and n-hexanol increase more; phenethyl alcohol can give bread a rose flavor, and n-hexanol has a grassy flavor. The content of acids in naturally fermented sourdough bread is also increased, especially acetic acid, which is the signature flavor in the bread core and is produced by heterotypic fermentation of lactic acid bacteria in the naturally fermented sourdough system. Aldehydes decreased, and esters increased as a result of the interaction between acids produced by lactic acid bacteria and alcohols produced by *Saccharomyces cerevisiae* in the naturally fermented sourdough system, which introduced ethyl linoleate and isoamyl acetate into the bread, giving the bread a creamy and banana flavor, respectively. Hydrocarbon compounds with a high threshold have little effect on flavor. Aromatic compounds increased after sourdough fermentation, as did heterocyclic and nitrogen-containing compounds. In addition, the flavor differences between the two naturally fermented sourdough breads are likely due to differences in their internal microflora structure. The difference in microorganisms between raisin sourdough and apple sourdough resulted in different flavor compound profiles of bread products.

Compounds	kind	Peak area/10 ⁷	kind	Peak area/10 ⁷
alcohol	11	1616.67	14	2274.32
acid	9	195.41	8	431.41
aldehyde	11	319.73	12	208.80
ester	12	111.23	16	204.12
ketone	7	181.24	6	377.29
hydrocarbon	4	43.98	4	73.37
aromatic compounds	3	150.75	5	174.05
Heterocycles and nitrogen-containing compounds	3	66.78	3	256.67

4. Conclusion

This study demonstrates that incorporating naturally leavened sourdough – derived from apples – significantly enhances the nutritional, flavor, and storage properties of bread. Raisin sourdough notably elevated the content of essential amino acids, increasing the proportion of essential amino acids. Furthermore, sourdough bread exhibited superior characteristics to other breads. These findings underscore the potential of natural sourdough as a multifunctional leavening agent, offering a sustainable strategy to produce nutrient-dense, flavorful, and shelf-stable bread while reducing reliance on commercial additives. Future applications could optimize sourdough microbiota to tailor amino acid profiles and flavor outcomes for artisanal baking innovations.

CRedit authorship contribution statement

Xuanyu Xing designed, performed experiments, and analyzed data.

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Declaration of Competing Interest

The authors declared no conflict of interest.

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