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Strategies to Reduce Acrylamide Formation in bread focusing on exogenous strain ferment sourdough

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ABSTRACT: Acrylamide (AM) is produced as a by-product of the Maillard reaction during the thermal processing of baked goods. Research has indicated that the addition of fermented sourdough significantly reduced the AM content in baked products. This study investigated the main metabolites of sourdough fermented by single strains of *Pediococcus pentosaceus* (PP) , *Saccharomyces cerevisiae* (ST), and their combinations. Additionally, the inhibitory effect of sourdough on AM in both bread and asparagine/glucose (Asn/Glc) chemical simulation system was examined, and the potential inhibitory mechanism was systematically elucidated. The key findings revealed that at an ST-to-PP ratio of 1:1, the two strains exhibited synergistic growth. The sourdough samples fermented with this mixed combination (MF-s) for 12 h exhibited the highest content of organic acids, resulting in a lower pH value than those of samples fermented with only ST-s or PP-s. The MF-s samples exhibited higher contents of maltose, glucose, fructose, sucrose, and total free amino acid than ST-s and PP-s. After 12 h of fermentation, the inhibition rate of AM in wheat bread and the Asn/Glc system reached the highest level. Particularly, MF-s exhibited the highest inhibition rate of AM (49.66%), followed by PP-s (42.23%) and ST-s (38.22%). These rates were significantly higher than those in the naturally fermented group ($P < 0.05$). The kinetic model analysis results indicated that the logistic exponential kinetic model was considered highly suitable for describing the kinetic process of AM.

Keywords: Acrylamide; *Pediococcus pentosaceus*; *Saccharomyces cerevisiae*; Sourdough; Bread.

1. Introduction

In recent years, the bakery industry has undergone rapid advancement owing to the rich flavor, nutritional benefits, and easy accessibility of baked goods. During the baking process, the carbohydrates in the food ingredients undergo the Maillard reaction at high temperatures. The Maillard reaction, which occurs during the baking process of goods, enhances flavor and texture but also yields certain potentially hazardous compounds [1], such as acrylamide (AM), furan, 5-hydroxymethylfurfural (5-HMF), and heterocyclic amines [2]. At the same time, it is easier to form AM in baked foods in the presence of asparagine. AM is a small, highly water-soluble chemical molecule with electrophilic groups. Studies have shown that the AM monomer can penetrate the skin, mucous membranes, respiratory system, and gastrointestinal tract. Food intake is considered one of the fastest and most efficient methods for the body to absorb AM [3,4]. After oral absorption, AM from food spreads widely in the body tissues via the bloodstream, reaching organs such as the

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brain, heart, liver, kidney, and breast milk [5]. This distribution process can pose risks to the body, potentially causing gene mutation and nervous system damage [3,6]. Therefore, exploring effective methods for regulating the AM content in baked goods to ensure their safety is crucial.

To reduce the final AM content at each stage of the baking process, several approaches can be implemented. These approaches include raw material pretreatment, physical methods (such as radio frequent heating and vacuum heating), chemical regulation methods (such as the addition of K^+, Na^+, Ca^{2+}, and Mg^{2+}), and incorporating glucoamylase, asparaginase, and other biological enzymes. However, these techniques have some drawbacks. For example, physical regulation methods and raw material pretreatment require time and skilled technical staff. These approaches may reduce the content of reactants, such as asparagine and reducing sugars [7,8], which can create unfavorable conditions for the Maillard reaction or minimize AM after its formation. However, studies have indicated that despite the use of cationic additives or enzymes to reduce the formation of AM in baked foods, the enzymes commonly used often hinder the activity of *Saccharomyces cerevisiae* in bread. This inhibition can result in reduced gas formation during the fermentation process and lower levels of starch hydrolysis and proteolytic activity. Moreover, excessive addition of additives can adversely affect food nutrition, sensory characteristics (including taste, texture, and color), and food safety [9]. The biological regulation approach utilizes exogenous strain fermentation technology to produce sourdough, which is then applied to baked items as a natural biological improver.

Despite acrylamide reportedly is present in relatively low amounts in existing bakery foods, especially in bread, therefore, there is a risk of ingesting acrylamide from eating bread. The addition of sourdough is beneficial in reducing the acrylamide content in bread. Fermentation by *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* is safe. Moreover, fermenting sourdough with exogenous strains provides numerous benefits as a natural biological enhancer [10]. For example, *Saccharomyces cerevisiae* and *Pediococcus pentosaceus* metabolize carbon sources in fermented sourdough to produce CO_2 and organic acids, resulting in a loose, porous texture and a unique mesh structure. This process significantly improves texture quality and promotes dough acidification [11]. Studies have indicated that the production of organic acids can effectively inhibit the protonation of reducing sugar amino groups and carbonyl groups, thereby reducing AM formation. Additionally, *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* deplete the nutrients such as carbon and nitrogen compounds in the dough during the fermentation of sourdough [12], which may reduce the contents of precursor compounds associated with AM production. Therefore, the use of *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* strains to prepare fermented sourdough as a natural biological enhancer may be a promising approach for reducing AM contents in foods.

This study examined the reduction of AM in bread and asparagine/glucose (Asn/Glc) simulation system using fermented sourdough produced by *Pediococcus pentosaceus* (JCM3586), *Saccharomyces cerevisiae* (CGMCC NO.3871), and their combination. Additionally, the study systematically investigated the inhibition of AM in wheat bread by sourdough fermented with specific strains and the underlying inhibition mechanism. The results of this study provide the bakery industry with an improved approach for reducing AM formation,

leading to the production of safe, natural, and acceptable bakery products and enhancing the utilization value of sourdough. Additionally, this study provides valuable guidance for ensuring the safety of baked goods and establishing AM-related food safety standards.

2. Materials and methods

2.1 Microorganisms and raw material

Pediococcus pentosaceus (JCM3586, Japan Collection of Microorganisms, PP); *Saccharomyces cerevisiae* (CGMCC NO.3871, ST) were provided by the State Key Laboratory of Dairy Biotechnology (Shanghai, China). Golden Statue bread flour (dry protein: 12.35, ash: 0.63, gluten: 35.7, moisture content: 11.47 g/100 g, the water absorption is 59%, formation and stabilization time of 1.27 and 8 min, respectively). Active dry yeast (Angle, China), sugar (Great Value, China), and edible salt (taikoo, China) were purchased from local supermarkets. Lactic acid, acetic acid, asparagine, maltose, glucose, fructose, sucrose, acrylamide, 5-hydroxymethylfurfural, 3-amino-propionamide standard (liquid grade) (Shanghai Gao Xin Glass Instrument Co., LTD.).

2.2 Microbial strains and culture conditions

The method referred to previous study[13].

Pediococcus pentosaceus were activated by two successive transfers in sterile MRS broth for 24 h using 5% (v/v) inoculums respective at 37℃. Cells were harvested by centrifugation (4500 g, at 4℃, 15 min), washed twice with sterile distilled water, and centrifuged (4500 g, at 4℃, 15 min). The *Pediococcus pentosaceus* fermentation broth was obtained.

Saccharomyces cerevisiae were activated by two successive transfers in sterile MRS broth for 24 h using 5% (v/v) inoculums respective at 30℃. Cells were harvested by centrifugation (4500 g, at 4℃, 15 min), washed twice with sterile distilled water, and centrifuged (4500 g, at 4℃, 15 min). The *Saccharomyces cerevisiae* fermentation broth was obtained.

2.3 Preparation of sourdough

The method referred to our previous study [14].

For experimental use, fresh cells (with 2.2 *Pediococcus pentosaceus* fermentation broth and *Saccharomyces cerevisiae* fermentation broth)were added immediately to the dough（wheat flour: pure water = 1:1.5）and mixed well, the dough is fermented at 30℃ for 24 h. (The initial inoculation count of fresh cells is about 8.0 logCFU/g sourdough). During this period, samples were taken every 4 hours to obtain a sourdough sample. After fermentation, the samples were freeze-dried and crushed in a freeze-drying machine. Sourdough samples were taken after fermentation and analyzed immediately for sourdough parameters. The colonies of lactic acid bacteria (LAB) and yeast in sourdough dough were counted by MRS and PDA.

Table1 Strain ratio of different mixed strains fermented sourdough

Group	Ratio(volume)
ST:PP	1:100
ST:PP	1:10

ST:PP	1:1
ST:PP	10:1

(NU-s: Natural sourdough (without exogenous fermentation strain); PP-s: *Pediococcus pentosaceus* sourdough; ST-s: *Saccharomyces cerevisiae-I* sourdough; MF-s: Mix strains soudough; PP(mix): PP in mix strains ; ST(mix): ST in mix strains)

2.3.1 Measurement of pH value, total titratable acid (TTA) and organic acids in sourdough

The pH value and TTA were determined in sourdough according to the following procedure [15]. The pH value was measured using a Titrando pH meter (Metrohm, Switzerland).A 10 g sample was dissolved in 90 mL of water and stirred for 10 minutes. The solution was titrated with 0.1 mol/L NaOH standard solution until reaching pH 8.5, and the volume of NaOH solution consumed (mL) was recorded. The TTA of sourdough was calculated based on the volume of NaOH solution needed to achieve pH 8.5.

The content of organic acids was determined using LC-20A system (Shimadzu, Kyoto, Japan). The freeze-dried sample (10 g) was dissolved in 90 mL of ultra-pure water, ultrasonically mixed at 60℃ for 15 min. Centrifuge the mixture at 4000 r/min for 20 minutes and filter the supernatant through a 0.22 μm filter. Set aside for future use. The chromatographic test conditions include a C18 column (4.60 mm × 250 mm, Agilent), a UV detector set at 210 nm, a mobile phase consisting of 10% methanol and 90% 0.02 mol/LKH_2PO_4 (v/v)with pH adjusted to 2.5 using phosphoric acid, a flow rate of 0.1mL/min, sample injection volume of 10 μL, and a column temperature of 20℃.

2.3.2 Analysis of reducing sugars and free amino acids in sourdough

The method for detecting reducing sugars followed the standards outlined in GB 5009.8-2016: National food safety standard Determination of fructose, glucose, sucrose, maltose and lactose in foods. After dissolving the sourdough and centrifugation, the samples were filtered through 0.45 μm syringe filters, and the resulting supernatant was analyzed using the HPLC-20 system (Shimadzu Corporation, Kyoto, Japan). The mobile phase was composed of acetonitrile and ultrapure water in a 7:3 ratio, and the flow rate of the mobile phase was 1.0 mL min^{-1}, with the column temperature maintained at 40℃ [17].The free amino acids in the sourdough samples were analyzed by Shiyanjia Lab (Shanghai, China) using the method established by Diana [18].

2.3.3 Fourier-transform infrared spectroscopy (FTIR)

The FT-IR spectra of freeze-dried samples were detected using an FT-IR spectrometer (Bruker, Germany). The freeze-dried sample was mixed with KBr at a ratio of 1:100 (w/w), and the mixture was then compressed into sheets. Infrared scanning was conducted over the range of 4000-500 cm^{-1}, with a resolution was 4 cm^{-1}, and the scanning was repeated 35 times. The infrared absorption map of the sample was recorded.

2.4 Bread making formulations

The method referred to our previous study [14].

Bread doughs were prepared by mixing all the ingredients (with 2.3 sourdough (NU-s, PP-s, ST-s, MF-s) replaced 30% of the flour (270 g), sugar (3.74%), active dry yeast (1.56%), table salt (1.00%) and water (58.89%). Bread was made for 3 min with a spiral mixer at low speed, followed by 8 minutes at medium speed

till dough shaped; then the dough was divided into 50 g loaves, shaped and placed on bakeware for 15 min. The loaves were then proofed in a proofer at 35℃ and 75% relative humidity for 60 min. Baking was carried out in heated oven at 185℃ for 25 min. The bread parameters were measured after cooling at room temperature.

(NU-b: Natural sourdough bread; PP-b: *Pediococcus pentosaceus* sourdough bread; ST-b: *Saccharomyces cerevisiae-I* sourdough bread; MF-b: Mix strains sourdough bread)

2.5 Construction of chemical simulation system for Asparagine/glucose (Asn/Glc)

The construction of the Asn/Glc simulation system can be attributed to Xu[19].

The equimolar asparagine(Asn) and glucose(Glc) were first weighed, with Asn weighing 6.606g and Glc 9.008g. The reactant powders used in the subsequent tests were carefully ground together to ensure sufficient surface area for the reactions. Weigh 0.43719g of the evenly ground powder, add 260 μL of sourdough pretreatment supernatant at different fermentation times.The reactant powders were oven-heated at 160℃ for 30 min and remove it every 5 minutes.

After heating, the reaction mixtures were immediately cooled in an ice bath and extracted with 10 μL sonication for 30min. The extracts were centrifuged at 4000 r/min for 30 min to obtain the extract of the final product of the reaction for the determination of acrylamide content.

2.6 Construction of Asn/Glc kinetic reaction system and acrylamide kinetic model

The sourdough, fermented for 12 h, was added to the Asn/Glc system. The reaction was then conducted at 160℃ for 0–50 min. The sample was removed every 5 min, and the AM content was determined [20–22].

(1) Logistic- growth curve model parameter values

$$C1(t)=\frac{A1}{1+\exp[k1(t1-t)]} \quad (1\text{-}1)$$

$$C2(t)=\frac{A2}{1+\exp[k2(t-t2)]} \quad (1\text{-}2)$$

(2) Logistic-Fermi model parameter values

$$C1(t)=\frac{a}{1+\exp[k1(t1-t)]}-\frac{a}{1+\exp(k1\times t1)} \quad (1\text{-}3)$$

$$C2(t)=\frac{1}{1+\exp[k2(t-t2)]} \quad (1\text{-}4)$$

$$C(t)=C1(t)\times C2(t) \quad (1\text{-}5)$$

(3) Logistic- index model parameter values

$$C1(t)=\frac{a}{1+\exp[k1(t1-t)]}-\frac{a}{1+\exp(k1\times t1)} \quad (1\text{-}6)$$

$$C2(t)=\exp(-\frac{t}{\tau}) \quad (1\text{-}7)$$

$$C(t)=C1(t)\times C2(t) \quad (1\text{-}8)$$

In the formula:

$C_1(t)$: function of acrylamide formation. $C_2(t)$: function of the elimination process of acrylamide. C(t): change of acrylamide content in the whole system. t: reaction time, τ: characteristic time; A_1 and K_1:

temperature dependence coefficients in the generation process. A_2 and K_2: the temperature dependence coefficients in the elimination process; t_1 and t_2: the inflection points of acrylamide formation and elimination.

2.6.1 AM measurement

The AM content in the sourdough bread was analyzed via HPLC (Shimadzu Management Co., Ltd., China), with reference to the method described by Andačić [23].

Samples were homogenized for 2 min in normal hexane (2 mL) then centrifugation (4500 g, 15 min), repeat twice. Then add Carrez I (1 mL), Carrez II (1 mL) and Ultrapure water (10 mL), After centrifugation (4500 g, 15 min), the water extracts were filtered through 0.22 μm syringe filters, and aliquots of the filtrates were analyzed by HPLC-20 system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV/visible detector (model SPD-20) at 208 nm; using a Shimadzu HPLC-20 infusion pump with the oven temperature of 35℃ and a flow rate of 0.8 mL min^{-1}.

$$\text{Inhibitory Rate} = \frac{C_0 - C}{C_0} \times 100\%$$

In the formula:

C_0: acrylamide generation amoun in control sample. C: acrylamide generation amoun in experimental group sample. The precision was calculated as the [(standard deviation/mean) × 100%].

2.6.2 Influence of fermented sourdough on redox potential in the Asn/Glc model

The simulated system was heated in an oven at 160℃ for 0–30 min, and then removed after every 5 min. Subsequently, 10 mL of ultra-pure water was added to the system, and the mixture was subjected to ultrasonic treatment for 30 min. Finally, the redox point of the system was detected by the PHSJ-3F pH meter detector (oustor , Shanghai, China).

2.6.3 Influence of 3-amino propionamide (3-APA) on the generation of AM in the Asn/Glc model

Initially, 100 μmoL of 3-APA standard was weighed, and 262 μL of water and sourdough solution were added to both the control and sourdough groups. The sample preparation was the same as 2.6.2, and the final product extract was obtained for the determination of acrylamide content. The determination method of acrylamide was referred to 2.6.1.

2.6.4 Influence of fermented sourdough on 5-HMF formation in the Asn/Glc model

The sample was treated in the same way as 2.6.1, and the final product extract was processed to obtain the final product extract for determining the HMF content, which was determined by HPLC, as described by Verma [24].

2.7 Statistical analysis

Three parallel samples were used for all experimental data, which were represented by mean ± standard deviation (SD). Data analysis was performed using GraphPad Prism Version 20.0.1 statistical Software (GraphPad Software Inc., San Diego, CA, USA). MATLAB software was used to construct the dynamic

model, and Pseudo-R^2 was used to evaluate the degree of fit of the model [25].

$$\text{Pseudo-}R^2=1-\frac{\text{SS Residual sum of squares}}{\text{SS Correct the sum of squares}} \quad (1\text{-}9)$$

3. Results and discussion

3.1 Growth characteristics of strain and acidification rate of sourdough

During sourdough fermentation with a Saccharomyces cerevisiae (ST)-to-Pediococcus pentosaceus (PP) ratio of 1:1 (Fig. 1a) , we found that the growth rate of PP is higher than ST within the first 0–8 h. The colony counts of PP rapidly increased from 7.33 to 8.68 (log CFU/g sourdough) ; the colony counts of ST increased from 7.32 (log CFU/g sourdough) to 8.39 (log CFU/g sourdough). In mixed strain-fermented sourdough from 8 to 24 h, both strains exhibited robust growth and featured synergistic capabilities. The trend in colony numbers observed in mixed strain-fermented sourdough was consistent with that in singly fermented sourdough. During the dough fermentation process with a compound ratio of 1:1 between PP and ST, PP and ST grew rapidly at 0-8 h, and tended to be stable at 12 h, which was shorter than other ratios. Therefore, we selected this ratio (ST:PP = 1:1) for mixed-strain dough fermentation and then measured the changes in certain metabolites during the fermentation process.

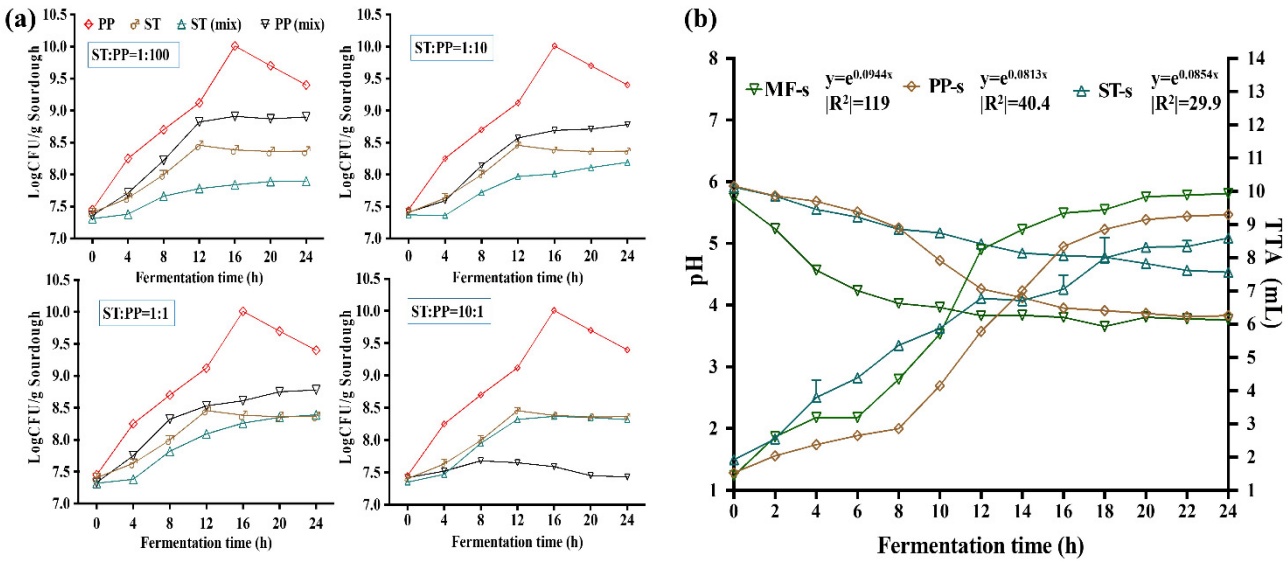

Figure 1 Fermentation characteristics of sourdough. (a) Growth status of *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* in fermented sourdough. (b) Changes in pH value and TTA during lactobacilli strains fermentation of sourdough.

To investigate the effect of yeast acidification rate on the AM content in wheat bread, the changing trend of pH value in sourdough under different fermentation times was examined to elucidate the regulatory effect of acidification rate on AM. The results (Fig. 1b) revealed that as the fermentation time increased, the pH value decreased from 5.93 to 3.76, while TTA increased from 1.44 to 9.93 mL. The pH value of fermentation for all strains was lower than that of natural fermentation, while TTA was higher for all types of fermentation.

The pH value of mixed bacteria (MF-s) tended to stabilize after 12 h, while the pH value of PP-s and ST-s decreased gradually during the entire fermentation process, indicating a slower acidification rate. MF-s ($|R^2|$ =119) exhibited a higher acidification rate than a single strain (the acidification rates $|R^2|$ of PP-s and ST-s

were 40.4 and 29.9, respectively). MF-s provided unique advantages to sourdough bread. During fermentation, MF-s produced organic acids through their metabolic activities, thereby reducing the pH value of the system.

Lactic acid and acetic acid were crucial contributors to the overall flavor profile of baked foods. *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* mainly achieved acidification during fermentation through the production of organic acids. Additionally, lactic acid and acetic acid played a key role in extending the shelf life of bread products [26].

Table 1 The change in lactic acid and acetic acid content in sourdough.

(mg/g sourdough)		0 h	4 h	8 h	12 h	16 h	20 h	24 h
Lactic acid	MF-s	1.182±0.079^{a}	10.09±0.021^{a}	13.25±0.014^{a}	15.20±0.011^{a}	15.89±0.015^{a}	16.20±0.076^{a}	16.3±0.057^{a}
	PP-s	1.090±0.037^{b}	9.014±0.010^{b}	12.24±0.025^{b}	14.28±0.015^{b}	14.46±0.051^{b}	13.43±0.017^{b}	13.38±0.024^{b}
	ST-s	0.984±0.016^{c}	3.941±0.051^{c}	5.646±0.053^{c}	6.439±0.091^{c}	7.299±0.049^{c}	6.615±0.018^{c}	6.052±0.054^{c}
Acetic acid	MF-s	0.539±0.068^{a}	1.342±0.089^{a}	1.566±0.015^{a}	1.708±0.011^{a}	1.897±0.055^{a}	1.692±0.0503^{b}	1.595±0.045^{b}
	PP-s	0.527±0.077^{b}	0.920±0.025^{c}	1.21±0.025^{c}	1.409±0.032^{c}	1.528±0.022^{c}	1.534±0.002^{c}	1.485±0.010^{c}
	ST-s	0.500±0.057^{c}	1.328±0.014^{b}	1.521±0.026^{b}	1.694±0.022^{b}	1.875±0.013^{b}	1.699±0.071^{a}	1.596±0.055^{a}

Note: Data are expressed as mean ± standard deviation (n=3), and different letters in the same column indicate significant differences ($P < 0.05$).

During the entire fermentation process, MF-s sourdough exhibited the highest total acid content. From 4 to 12 h, lactic acid and acetic acid in MF-s were rapidly produced and gradually accumulated. Consequently, the contents of lactic acid and acetic acid in sourdough increased to 16.19 and 2.70 mg/g sourdough, respectively. These values were significantly higher than those of PP-s (lactic acid and acetic acid contents of 13.52 and 1.40 mg/g sourdough, respectively) and ST-s (lactic acid and acetic acid contents of 6.20 and 1.60 mg/g sourdough, respectively) in single-strain fermentation. An increase in organic acid content, a decrease in pH value, and an increase in TTA in the mixed strains of fermented sourdough were mainly attributed to the combination of two strains during fermentation.

3.2 Measurement of reducing sugar and free amino acid content in sourdough

The changing trend of reducing sugars and amino acids in sourdough was examined to elucidate the relationship between the content of precursors and the formation of AM. Glucose and fructose were the main reducing sugars in baked foods, with maltose present in small amounts, and the ratio of glucose to fructose was a significant factor to consider. As the fermentation time prolonged, the maltose, glucose, fructose, and sucrose contents in sourdough decreased, reaching their lowest levels (Fig. 2). The reduced sugar contents in sourdough after 24 h fermentation was similar to that observed during the early stages of fermentation. With extended fermentation time, MF-s exhibited a lower reducing sugar content than NU-s, ST-s, and PP-s. This can be attributed to the accumulation of organic acids produced by ST and PP, which led to a decrease in pH values and an increase in the activities of amylase and glucoamylase, thereby promoting the decomposition of flour [27].

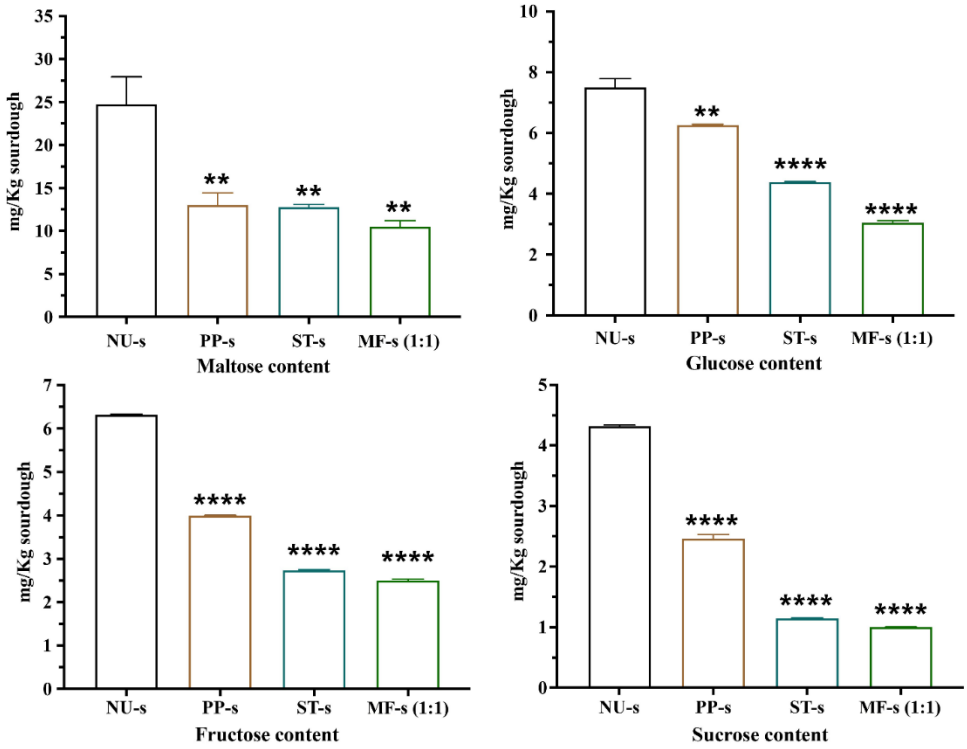

Figure 2 Determination of reducing sugar in fermented sourdough.

Figure 3a and b shows the FTIR spectra of reducing sugar (Glc) and asparagine (Asn) in MF-s and NU-s after fermentation for 12 h. Significant differences were observed in the FTIR spectra between natural fermentation and mixed fermentation. However, the characteristic absorption peaks remained similar in the range of 4000–500 cm^{-1} before and after fermentation, indicating that the strain-fermented sourdough did not affect the main functional groups of reducing sugar [28]. 3300cm^{-1} is the tensile vibration peak of -OH in C-OH, 1650cm^{-1} absorption peak is the C=O asymmetric tensile vibration in -CHO, and the absorption peak within 1100cm^{-1} further indicates that the sample is glycocompounds. The characteristic peak near 980cm^{-1} is the characteristic absorption peak of the end-based carbon on the saturated carbon. Moreover, before and after fermentation, the transmittance of characteristic peaks in the spectrum of reducing sugar decreased. This indicates that fermentation stimulated both the generation and degradation of reducing sugar, further reducing its content in the sample.

The spectrum of asparagine (Fig. 3b) exhibited an absorption peak at 3100 cm^{-1}, corresponding to the tensile vibration peak of $-NH_2$ in $-CONH_2$. Additionally, the absorption peak in the range of 1200−1700 cm^{-1} indicated the presence of an amide compound in the sample. The characteristic absorption peaks remained similar in the range of 4000–500 cm^{-1} before and after fermentation [29], indicating that fermentation of sourdough by the strain did not affect the major functional groups of amide groups.

Table 2 shows the changes in the amino acid content of sourdough resulting from the mixed fermentation of PP and ST compared with their individual fermentation. Overall, the fermented sourdough exhibited higher free amino acid content than the control group. After 12 h fermentation, the content of total free amino acids in MF-s reached the highest level. Studies have identified amino acids most associated with AM production, including asparagine, glutamine, lysine, arginine, and amino acids containing imines such as histidine and

tryptophan. The contents of aspartic acid, glutamic acid, lysine, arginine, and histidine in MF-s increased with prolonged fermentation time. Asparagine and glutamine can undergo amination and deamination to form aspartic acid and glutamic acid. Protein degradation led to an increase in aspartic acid and glutamic acid, thereby reducing the contents of asparagine and glutamine. Consequently, the formation of AM from precursor compounds decreased.

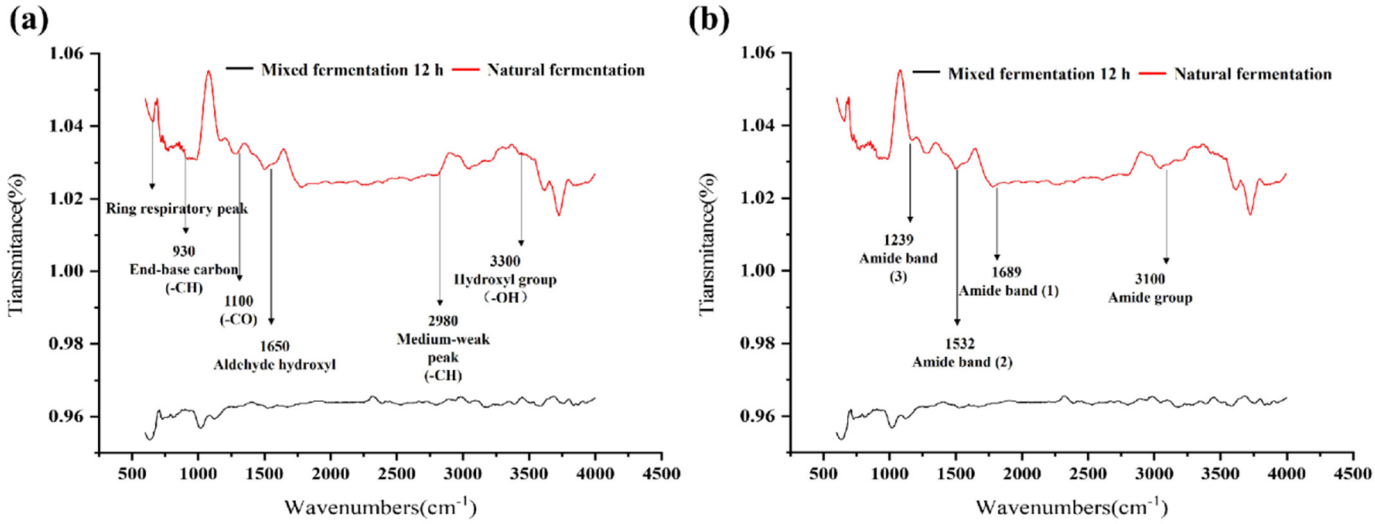

Figure 3 (a) Fourier infrared determination of reducing sugar in fermented sourdough; (b) Fourier infrared determination of asparagine in fermented sourdough.

Table 2 Free amino acid content of fermented sourdough.

Content (g/100g sourdough)	Control	NU-s			ST-s			PP-s			MF-s		
	unfermented	8 h	12 h	16 h	8 h	12 h	16 h	8 h	12 h	16 h	8 h	12 h	16 h
Aspartic acid	0.269	0.305	0.318	0.306	0.327	0.354	0.39	0.334	0.358	0.432	0.328	0.450	0.408
Glutamic acid	0.249	0.256	0.364	0.183	0.18	0.198	0.364	0.308	0.352	0.373	0.262	0.396	0.478
Lysine	0.092	0.089	0.213	0.124	0.14	0.174	0.228	0.103	0.147	0.227	0.094	0.218	0.238
Arginine	0.097	0.135	0.035	0.152	0.132	0.165	0.035	0.108	0.102	0.015	0.126	0.061	0.04
Histidine	0.051	0.073	0.071	0.062	0.088	0.117	0.071	0.104	0.116	0.073	0.083	0.062	0.073
Alanine	0.091	0.109	0.086	0.22	0.162	0.167	0.086	0.091	0.101	0.086	0.089	0.104	0.091
Methionine	0.378	0.313	0.328	0.475	0.458	0.428	0.458	0.356	0.353	0.47	0.333	0.402	0.398
Threonine	0.078	0.148	0.105	0.124	0.209	0.131	0.105	0.11	0.103	0.107	0.113	0.155	0.12
Valine	0.095	0.089	0.179	0.126	0.123	0.142	0.199	0.15	0.165	0.191	0.14	0.177	0.191
Total content	**1.4**	**1.517**	**1.699**	**1.772**	**1.819**	**1.869**	**1.936**	**1.664**	**1.797**	**1.992**	**1.568**	**2.025**	**2.037**

3.3 The content of acrylamide (AM) in sourdough fermentation bread

Compared with naturally fermented bread (NU-bread), the AM content in bread fermented by *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* decreased (Fig. 4a). The most significant reduction in AM content occurred within 8–12 h of fermentation. Particularly, the NU-bread exhibited an AM content of 283.30 μg/kg. MF-b exhibited the highest reduction rate of AM, with a decrease of 55.81%, followed by PP-b and ST-b, which decreased by 48.73% and 42.22%, respectively.

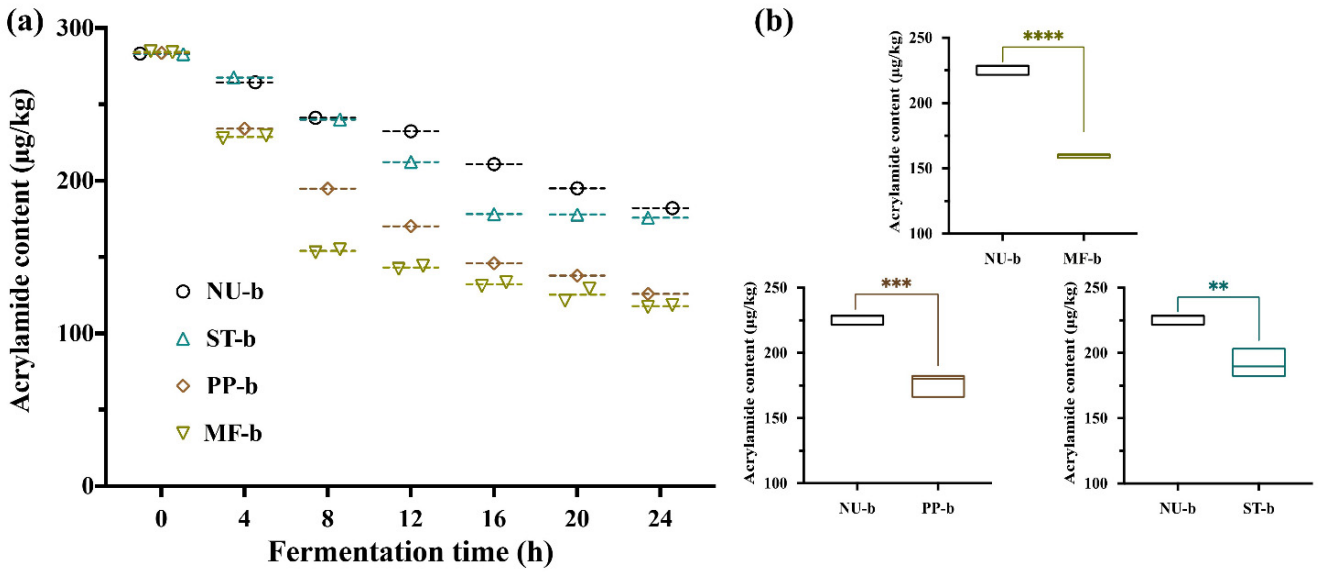

Figure 4 Acrylamide content (a) Acrylamide levels in bread samples prepared with sourdough. (b) The relationship of acrylamide in different fermentation groups. The *Error bars* represent ± SD. ****: $P < 0.001$, ***: $P < 0.005$, **: $P < 0.01$, *: $P < 0.05$.

Bread fermented by mixed strains exhibited a higher reduction rate of AM than bread fermented by single strains. Fermentation are more effective to reduce AM due its antioxidant potential and organic acids profile. Mixed strains can produce numerous organic acids, thereby reducing the pH value of the sourdough environment and inhibiting the formation of AM in food. Additionally, mixed strain fermentation inhibited the formation of AM by consuming precursor materials (such as protein and reducing sugar) [9].

3.4 Kinetics of AM generation and elimination in asparagine/glucose simulation system

The results shown in Fig. 5a illustrate the concentration–inhibition rate relationship of sourdough fermented by strains on AM in the Asn/Glc system. The selected strain of fermented sourdough can reduce the AM content in the Asn/Glc system across different fermentation times. The inhibition rate of AM ranged from 3.67% to 49.67%. With prolonged fermentation time, the inhibition rate of AM on fermented sourdough gradually increased. After 12 h of fermentation, the maximum inhibition rates of AM reached 32.91%, 45.42%, 40.66%, and 49.67%. Among the four types of fermented sourdough, MF-s exhibited the highest inhibitory rate of AM, followed by PP-s, with NU-s featuring the lowest inhibitory rate.

The optimal fermentation time (12 h) of sourdough was selected for the kinetics study. During the initial fermentation stage, the AM content rapidly increased and then decreased after reaching the maximum, indicating a dynamic process of formation and elimination of AM in the Asn/Glc system (Fig. 5b). Compared with NU-s, the addition of 12-h strain-fermented sourdough reduced the AM content in the Asn/Glc system. Consequently, the AM content in the MF-s group decreased by 42.44%, while PP-s and ST-s decreased by 31.63% and 20.90%, respectively. Additionally, compared with NU-s, the time required to reach the maximum production of AM in the strain-fermented sourdough group was delayed to a certain extent. Particularly, the time required to reach the maximum production of AM in MF-s, PP-s, and ST-s groups was 35, 30, and 30 min, respectively. This result indicates that the fermentation of sourdough by all

strains could inhibit AM formation. However, the peak inhibitory effect was observed at the midpoint of the reaction.

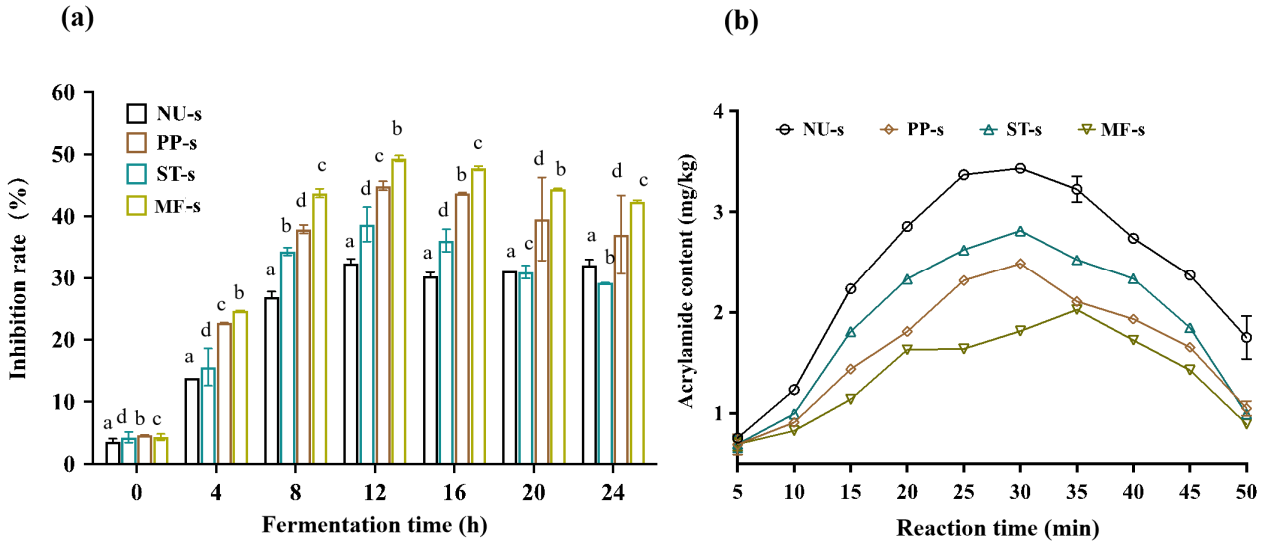

Figure 5 (a) Inhibition rate of acrylamide in Asn/Glc simulation system by Fermented sourdough. (b) Effect of Fermented sourdough on formation and elimination of acrylamide in Asn/Glc system.

3.5 Fitting of kinetic response model in the Asn/Glc simulation system

The logistic growth curve model was fitted by classifying the production process of AM into two stages, namely formation and elimination. The results indicated that the pseudo-R^2 values for both the formation and elimination processes exceeded 0.97 (Table 1), except for MF-s, indicating a high degree of fitting for the logistic growth curve model. However, previous research has indicated that in the simulation system, AM formation and elimination occurred simultaneously throughout the entire reaction process. The time parameter t_2 for the elimination process was significantly inconsistent with the inflection point time in the aforementioned kinetic curve. Therefore, the logistic growth curve model was considered inadequate for representing the reaction process. Consequently, this model was discarded.

The logistic-Fermi model was designed to simulate the formation and elimination of AM as simultaneous processes. The fitting results revealed that all the pseudo-R^2 values exceeded 0.92 (Table 1). However, in the control group and the models of PP-s and MF-s groups, the standard deviation of parameter "a" was excessively large. Additionally, the time parameter t2 represented the inflection time point in the elimination process. However, the obtained result for parameter t2 was significantly inconsistent with the inflection time point observed in the dynamic curve obtained previously. Consequently, the logistic-Fermi model was disregarded.

Table 3 Fitting of kinetic response model in Asn/Glc simulation system

1. Logistic- growth curve model parameter values					
Asn/Glc parameter		Control group	PP-s	ST-s	MF-s
Formation process	A_1	5.02 ± 0.51	2.77 ± 0.51	3.64 ± 0.50	2.13 ± 0.39
	K_1	0.15 ± 0.02	0.13 ± 0.05	0.16 ± 0.04	0.11 ± 0.06
	t_1	12.41 ± 1.82	9.11 ± 3.63	10.17 ± 2.45	6.65 ± 4.13
	Pseudo-R^2	0.9848	0.9232	0.9485	0.8282
Elimination process	A_2	5.40 ± 2.88	2.62 ± 0.47	3.42 ± 0.25	2.58 ± 1.68

K_2	0.10 ± 0.08	0.12 ± 0.06	0.16 ± 0.03	0.11 ± 0.10
t_2	37.90 ± 12.05	42.60 ± 4.03	40.21 ± 1.49	39.59 ± 14.70
Pseudo-R^2	0.9867	0.9898	0.9967	0.9727
2. Logistic-Fermi model parameter values				
Asn/Glc parameter	Control group	PP-s	ST-s	MF-s
a	179.30 ± 93.44	6.24 ± 0.49	10.39 ± 7.60	7.37 ± 0.20
K_1	0.07 ± 0.06	0.10 ± 0.00	0.11 ± 0.08	0.08 ± 0.00
t_1	60.00 ± 54.28	4.90 ± 4.13	4.06 ± 1.38	0.01 ± 0.02
K_2	0.09 ± 0.04	0.11 ± 0.00	0.15 ± 0.03	0.09 ± 0.01
t_2	13.04 ± 18.44	39.22 ± 1.45	37.14 ± 7.41	33.61 ± 1.66
Pseudo-R^2	0.9850	0.9766	0.9863	0.9204
3. Logistic- index model parameter values				
Asn/Glc parameter	Control group	PP-s	ST-s	MF-s
a	63.30 ± 21.22^{a}	342.6 ± 57.20^{b}	91.05 ± 5.59^{a}	44.89 ± 6.89^{a}
K_1	0.15 ± 0.01^{a}	0.13 ± 0.00^{a}	0.14 ± 0.00^{a}	0.11 ± 0.00^{d}
t_1	24.32 ± 2.27^{a}	28.21 ± 1.13^{c}	27.45 ± 0.22^{b}	31.57 ± 0.27^{d}
τ	12.74 ± 2.70^{a}	9.21 ± 0.28^{ab}	10.42 ± 0.18^{ab}	12.76 ± 0.76^{a}
Pseudo-R^2	0.9860	0.9572	0.9867	0.9763
4. Correlation between predicted and actual values of acrylamide				
Correlation Coefficient	Control group	PP-s	ST-s	MF-s
R^2	0.9622	0.9742	0.9696	0.9754

Note: Data were expressed as mean ± standard deviation (n=3), and different letters in the same line indicated significant differences ($P < 0.05$).

In the logistic index model, the pseudo-R2 values of all experimental groups exceeded 0.95 (Table 1), indicating a high model fit. The parameters k_1 and t_1 of MF-s were significantly lower and higher than those of the control group ($P < 0.05$), respectively. This suggests that the addition of MF-s could significantly inhibit the formation of AM during the initial stage and considerably increase the time required for the AM content to reach its peak. Parameter t_1 of PP-s and ST-s significantly exceeded that of the control group ($P < 0.05$), while PP-s and ST-s exhibited lower k_1 than the control group but showed no significant difference ($P > 0.05$). This indicates that the addition of PP-s and ST-s to the sourdough could significantly inhibit AM formation. Although the time required for the AM content in the fermented group to reach its maximum increased, the difference compared with the control group was not significant. No significant difference was observed in parameter τ of all experimental groups compared with the control group ($P > 0.05$).

The predicted values were calculated using the established simulation equation, and a correlation scatter plot was created to compare the forecasted and actual values of AM, as shown in Table 1(4). A strong correlation coefficient exceeding 0.9 between the actual and anticipated values of AM suggested a strong alignment with the logistic index model.

3.6 Effect of fermented sourdough on the chemical reaction of asparagine/glucose simulation system

Numerous investigations have revealed that the elimination of AM may be achieved through the polymerization of AM caused by oxidative free radicals. The oxidation–reduction potential (ORP) can change in response to alterations in oxidation free radicals. ORP increased with extended reaction time (Fig. 6a). The addition of MF-s resulted in the most significant increase in ORP, followed by PP-s and ST-s, which had lower organic acid contents. These findings suggest that MF-s addition could significantly enhance the increase in ORP of MR induced by heating. Therefore, one possible approach to promote the removal of AM may involve increasing the positive voltage of the Maillard reaction system [30,31].

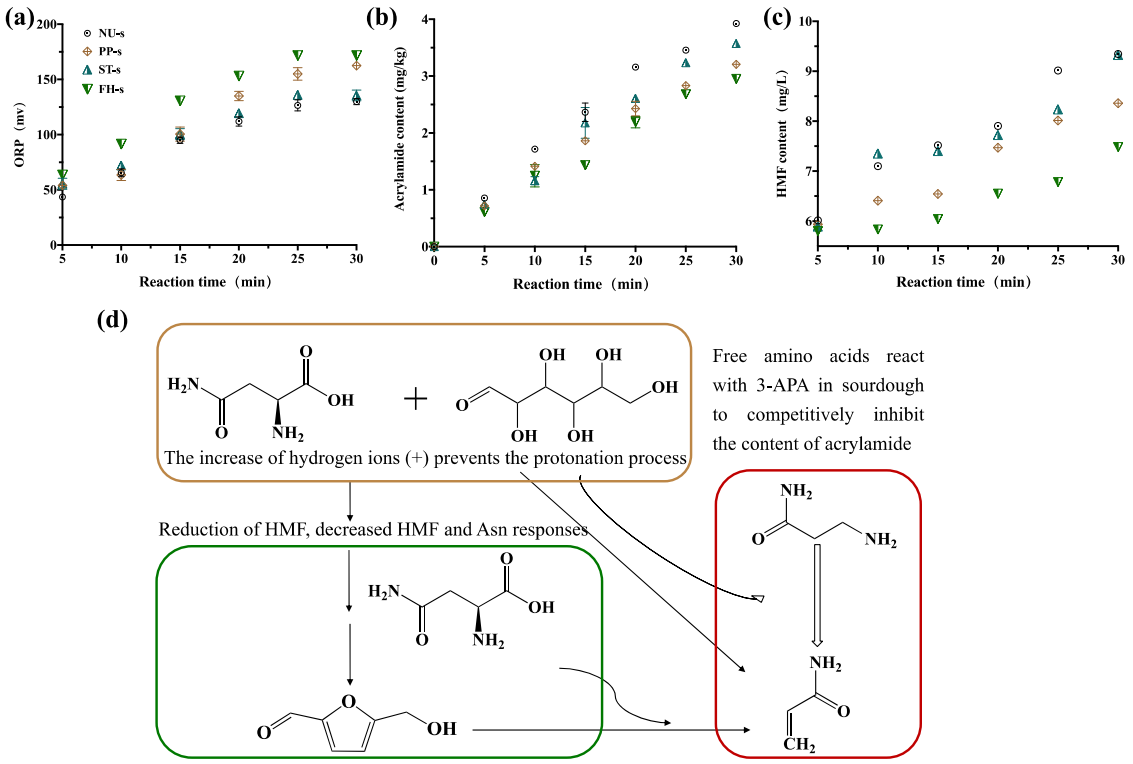

Figure 6 Effect of fermented sourdough on the chemical reaction of asparagine/glucose simulation system: (a) Effect of fermented sourdough on ORP potential in asparagine/glucose simulation system. (b) The result of 3-APA formation of acrylamide in the asparagine/glucose simulation system by fermented sourdough. (c) Effect of fermented sourdough on 5-hydroxymethylfurfural formation in asparagine/glucose simulation system. (d) Summary of intervention mechanism of mixed bacteria fermented sourdough on formation of acrylamide.

Additionally, 3-APA was considered a crucial direct precursor of AM formation, as it converted to AM after deamination [32]. To simulate the reaction system and assess the change in AM contents, sourdough fermented for 12 h was combined with 3-APA, which can elucidate whether the inhibition of AM formation in the 12-h fermented sourdough involved the transformation path of 3-APA. The AM content in the NU-s group continuously increased with extended reaction time (Fig. 6b). After the reaction lasted for 15 min, the content of 3-APA in the MF-s group significantly decreased compared with that of NU-s (30.64%). These results reveal that 3-APA was converted to AM under heating conditions and that the production of AM increased with the extension of reaction time. The strain-fermented sourdough could inhibit AM formation by inhibiting the conversion of 3-APA to AM. This inhibition could be attributed to certain amino acid components in the sourdough strain produced by the strain, which can react with 3-APA and consume it, thereby reducing the amount of 3-APA available for conversion to AM.

HMF, a product of the Maillard reaction, competed with glucose for asparagine and contributed to the generation of more AM [33]. This indicates that the presence of HMF enhanced the formation of the Maillard reaction system, thereby promoting the AM formation. With the extension of reaction time, the HMF content increased in both the naturally fermented sourdough and the strain-fermented sourdough groups (Fig. 6c). However, the increase in HMF was significantly lower in the strain-fermented sourdough than in the naturally fermented sourdough, particularly during the middle and late stages of the reaction (20–30 min). These results indicate that the addition of fermented sourdough inhibited HMF formation in the Asn/Glc system. The

inhibitory effect of PP-s and ST-s was stronger than that of NU-s. Moreover, the inhibitory effect of MF-s was the strongest but not significantly different from PP-s ($P > 0.05$). The result was consistent with the trend in AM production (Fig. 3), indicating that strain-fermented sourdough could hinder AM formation by inhibiting HMF formation.

4. Conclusions

The use of strains in fermentation emerged as an effective approach for reducing AM in baked foods. This reduction resulted from increased levels of acidification rate and reduced content of precursor compounds. The results of this study indicated that mixed strains could significantly reduce AM levels in both bread and simulation systems. In all scenarios, the AM content in both wheat bread and simulation systems was lower than that in the control group, with reductions of 55.81% and 49.67%, respectively, through sourdough fermentation. Moreover, the reduction potential of AM through mixed fermentation exhibited strain specificity and varied according to the strain ratio. At a 1:1 ratio of the two strains, the reduction potential of AM was optimized. These results can be attributed to the combined effects of increased acidification rate and reduced contents of preconditioned materials. The decrease in AM formation during both single and mixed fermentation of sourdough occurred through two main mechanisms. First, a reduction in related precursor compounds inhibited the progress of the Maillard reaction: the reduction of reducing sugar content, the increase of amino acid components competitively inhibited the formation of 3-APA to acrylamide and the formation of HMF was reduced thereby reducing the precursor substances related to AM formation; second, fermentation processes hindered the protonation reaction involved in AM formation and the accumulation of organic acids promotes its elimination. Additionally, this study provides valuable guidance for ensuring the safety of baked goods and establishing AM-related food safety standards.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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